CIRCADIAN CONTROL OF IMMUNITY

EDITED BY: Koichi Ikuta and Christoph Scheiermann
PUBLISHED IN: Frontiers in Immunology

IUIS

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-432-0
DOI 10.3389/978-2-88966-432-0

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CIRCADIAN CONTROL OF IMMUNITY

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We acknowledge the initiation and support of this Research Topic by the International Union of Immunological Societies (IUIS). We hereby state publicly that the IUIS has had no editorial input in articles included in this Research Topic, thus ensuring that all aspects of this Research Topic are evaluated objectively, unbiased by any specific policy or opinion of the IUIS.

Citation: Ikuta, K., Scheiermann, C., eds. (2021). Circadian Control of Immunity. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-432-0

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frontiers in Immunology

EDITORIAL
published: 30 November 2020
doi: 10.3389/fimmu.2020.618843

Editorial: Circadian Control of Immunity

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Keywords: circadian immunity, innate immunity, adaptive immunity, leukocyte migration, control of circadian immunity

Editorial on the Research Topic

Circadian Control of Immunity

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *18 October 2020*
Accepted: *22 October 2020*
Published: *30 November 2020*

Citation:
Ikuta K and Scheiermann C (2020) Editorial: Circadian Control of Immunity. Front. Immunol. 11:618843. doi: 10.3389/fimmu.2020.618843

INTRODUCTION

The circadian clock influences virtually every aspect of life in mammals. Circadian clocks allow the organism to adjust to and anticipate recurring changes in their rhythmic environment, allowing for a better fitness and survival (1, 2). Within the immune system, it has long been known that the organism's response to immune stimulation is highly time-of-day dependent, resulting in over-activation and even death (3). Thus, while the existence of an overall oscillation in the response to an immune stimulant over 24 h has been recognized decades ago, the molecular mechanisms behind these features remained elusive until recently. In this special issue, we highlight the recent developments in the fast-growing field of circadian immunology.

Historically, the influence of the circadian clock on the innate system has been recognized first and thus this is the aspect of circadian immunology that we know most about so far. This is also due to the fact that time-of-day was thought to exert its influence most strongly on the acute effect of the immune system. More recent data, however, demonstrate that also the adaptive immune system is clock-controlled, although also here previous observations had already indicated an impact of time-of-day (4). This review series focuses on the recent developments in the circadian aspects of immune cell functions, providing an overview over the innate and adaptive immune system, neuronal and hormonal control as well as the influence of the microbiome on rhythmic immunity.

One of the most prominent features of a rhythmic immune system is the rhythmic fluctuation of immune cells in blood. Recent data indicate that this reflects their redistribution from blood to tissues, which is reviewed by Yuan et al. Thus, the temporal difference in the presence of certain immune cells at specific sites in blood and tissues will certainly contribute to any differences in the immune response.

RHYTHMS IN THE INNATE AND ADAPTIVE IMMUNE SYSTEM

The cell type that so far has been investigated in most detail is the macrophage (5, 6). Timmons et al. provide an in-depth overview over the recent developments with respect to their time-of-day dependency. Neutrophils, the most abundant immune cells in human blood, have remained a much less-studied leukocyte subset but have also been shown to be highly rhythmic in their activity and their trafficking patterns (7, 8). Aroca-Crevillén et al. provide an overview into the recent scientific developments in this aspect. Pourcet and Duez give insights into the rhythmic activation of the inflammasome, a key inflammatory signalling complex that integrates inflammatory input with immune cell output (9). In addition, the adaptive immune system is highly rhythmic and this has been demonstrated in detail with respect to allergic reactions. Nakao sums up the recent data into the role of the circadian clock in allergy.

NEURAL AND HORMONAL CONTROL

An important question that is currently an active field of research is how immune cells and immune responses in general are entrained. Recent data indicate that both glucocorticoids (10) as well as the sympathetic nervous system (11, 12) can govern these oscillations. Leach and Suzuki as well as Shimba and Ikuta discuss these recent developments with respect to adrenergic nerves as well as glucocorticoids, respectively. Interestingly, these oscillations are not only affecting mature leukocyte populations but are also observed at the level of hematopoietic stem and progenitor cells (13). García-García and Mendez-Ferrer discuss the recent development in the field with respect to immature hematopoietic cell populations.

MICROBIOTA

While the field is currently trying to better understand how a complex, multicellular organism orchestrates rhythmic immune reactions, the level of complexity is increased even further by the role that exogenous factors, predominantly the commensal microbiota, play in this. Aspects of the gut microbiota have been shown to be strongly rhythmic (14) and Butler and Gibbs, as well as Kubo sum up the recent insight into this role.

CONCLUSIONS

The circadian control of immunity is achieved in a cell autonomous manner by clock genes and can be entrained with the help of adrenergic nerves and glucocorticoids. This collection of review articles on the Research Topic "Circadian Control of Immunity" provides the latest and comprehensive update in this rapidly growing field of immunology research.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Work in KI's lab is supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers 20K21525 and 18K15184. Work in CS's lab is supported by the Swiss National Science Foundation (SNF, 310030_182417).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 14 February 2020
doi: 10.3389/fimmu.2020.00102

A Tissue-Specific Rhythmic Recruitment Pattern of Leukocyte Subsets

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The circulating of leukocytes in the vasculature to reach various organs is a crucial step that allows them to perform their function. With a sequence of interaction with the endothelial cells, the leukocytes emigrate from the circulation either by firm attachment to vascular beds or by trafficking into the tissues. Recent findings reveal that the leukocyte recruitment shows time as well as tissue specificity depending on the cell type and homing location. This spatiotemporal distribution of leukocyte subsets is driven by the circadian expression of pro-migratory molecules expressed on the leukocytes and the endothelium. Both the systemic circadian signals and the cell's intrinsic molecule clock contribute to the oscillatory expression of pro-migratory molecules. The rhythmic recruitment of leukocytes plays an important role in the time-dependency of immune responses. It also helps to update blood components and maintain the tissue circadian microenvironment. In this review, we discuss the current knowledge about the mechanisms of the circadian system regulating the leukocyte rhythmic migration, the recruitment pattern of leukocyte subsets into different tissue/organs, and the time-dependent effects behind this process.

Keywords: circadian rhythm, leukocyte, recruitment, function, chronopharmacology

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *03 November 2019*
Accepted: *15 January 2020*
Published: *14 February 2020*

Citation:
Yuan Y, Wu S, Li W and He W (2020) A Tissue-Specific Rhythmic Recruitment Pattern of Leukocyte Subsets. Front. Immunol. 11:102. doi: 10.3389/fimmu.2020.00102

INTRODUCTION

Blood leukocyte numbers display circadian rhythms in various mammalian species, like rodents (1) and human (2), with a consistent trend, showing a peak in the resting phase and a trough in the activity phase for most of the leukocyte subsets (3). This process reflects the dynamic emigration of leukocytes from the bone marrow (4) and the recruitment to various organs (3). It has been proved that the major leukocyte subsets, including neutrophils, inflammatory monocytes, non-inflammatory monocytes, CD4 T cells, CD8 T cells, NK cells, and eosinophils, emigrate from the mouse's blood stream and recruit into distinct tissue/organs in a rhythmic manner with the highest homing occurring at the rest-activity transition phase (3). However, the mechanism that governs the tissue-specific rhythmic recruitment pattern and the time-dependent effects brought by this process are not entirely understood.

THE RHYTHMIC EMIGRATION OF LEUKOCYTES IS GOVERNED BY THE PRO-MIGRATORY MOLECULES

The leukocyte migration occurs by intensive interaction of adhesion molecules and chemokine receptors with endothelial cells in multiple steps, including adhesion, rolling, crawling, and transmigration (5). Through a time-based screening approach, the expression oscillations of adhesion molecules and chemokine receptors on the surface of different leukocytes and vascular beds in mice have been identified (3, 6) (**Figure 1A**). Blocking receptor–ligand interactions on the endothelium (ICAM-1 or VCAM-1) or the leukocytes (CXCR4, L-selectin, CD11a, or CD49d) abolishes the rhythmicity of leukocyte emigration from murine blood, suggesting that both leukocytes and the microenvironment contribute to the time-dependent homing process (3). Moreover, the optimal efficacy of those migration blockers is acquired at the rest-activity transition time point when ICAM-1, VCAM-1, CXCR4, and CD49d reach their peak expression levels (3), indicating that the circadian recruitment of leukocytes is dependent on the oscillatory expression of pro-migratory molecules. In addition to this time-dependent feature, the expression of pro-migratory molecules exhibits leukocyte and tissue specificity. Different mouse leukocyte subsets display a rhythmic homing profile to distinct organs, which can be inhibited by targeting specific pro-migratory molecules (**Figure 1A**) (3). Consequently, these data implicate that the pro-migratory molecules with time- and tissue- specific expression signatures form a homing code that guides the homing of different leukocyte subsets.

THE CIRCADIAN SYSTEM REGULATES LEUKOCYTE RHYTHMIC HOMING

The circadian rhythm is self-sustained, which is primarily attributed to the autonomous molecular clock consisting of a transcription–translation feedback loop. Briefly, two central transcription factors, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle Arntl-like protein 1, also known as ARNTL), form a heterodimer and bind to Enhancer Boxes (E-box) containing sequences to induce the expression of clock-controlled genes, including their negative regulator *Per1/2/3* (period circadian protein homologs) and *Cry1/2* (cryptochromes), which inhibit further transcriptional activation by CLOCK/BMAL1 complex. An accessory feedback loop involves REV-ERBα and REV-ERBβ proteins (encoded by *Nr1d1* and *Nr1d2*), which compete with ROR proteins to inhibit *Bmal1* transcription via Rev-Erb/ROR response elements (RORE) (7, 8). The circadian genes can modulate leukocyte migration by regulating pro-migratory molecules. *Bmal1* is the major target for diminishing circadian rhythms as the single knockout induces molecular and behavioral arrhythmicity in mouse (8). B-cell (*CD19-cre*) and neutrophil (*Lyz2-cre*)-specific *Bmal1* deletion abolish the time-of-day differences in the expression of CD11a on B cells and PSGL-1 on neutrophils, thus ablating their homing rhythmicity to the spleen (3). Endothelial cell-specific *Bmal1* knockout (*Cdh5–cre*ERT2: *Arntl flox*) mice lose the circadian expression of VCAM-1 and ICAM-1 on the endothelium of the lung and liver, respectively, resulting in the arrhythmic homing of leukocytes to these two organs (3). With chromatin immunoprecipitation assays, BMAL1 has been found to bind to the neutrophil E-box element of *Cxcl2* to increase its expression, which induces neutrophil aging (9) that have higher expression levels of CD11b and CD49d (10). CLOCK binds to an E-box-like enhancer of *Icam-1* and regulates the adhesion of mononuclear cells to endothelial cells by increasing the expression of ICAM-1 and adhesion related genes on the cultured endothelial cells (11). In addition to BMAL1 and CLOCK, other proteins involved in the molecule clock loop also play a role in regulating the expression of pro-migratory molecules. Rev-erbα binds to the RORE of *Ccl2* to repress its expression in mouse macrophages and impairs cell adhesion and migration (12). Overexpression of *Cry1* reverses the increased VCAM-1, ICAM-1, and E-selectin expression on the vascular endothelial cells in sleep deprivation mice and suppresses the binding of monocytes to endothelial cells (13). These studies further support that the circadian clock genes regulate the oscillatory expression of pro-migratory molecules and modulate leukocytes migration.

The peripheral clocks are synchronized by the suprachiasmatic nucleus (SCN) located in the hypothalamus to be phase coherent with the environment. The environment light conditions are transferred into photic neural input by the eye and transmitted to the SCN central clock via the retinohypothalamic tract (14). This timing information is further transmitted from the SCN to the peripheral clocks through two major pathways, neural and humoral signals (8); both can influence the rhythmic recruitment process of leukocytes through regulating pro-migratory molecules. The sympathetic nerves act on a β-adrenoreceptor to synchronize the emigration of mouse leukocytes to the bone marrow and muscles by inducing tissue-specific oscillation of endothelial cell adhesion molecules and chemokines (P-selectin, E-selectin, and VCAM-1 in the bone marrow as well as ICAM-1 and CCL2 expression in the skeletal muscle) (6). The increased nocturnal homing of mouse leukocytes to the bone marrow is mediated by increased expression of E-selectin and VCAM-1 on the endothelium, which are controlled by the interplay between cholinergic signals and sympathetic noradrenergic tone (15). In terms of the hormone pathway, higher cortisol level in the morning increases the CXCR4 expression of human CD4 T cells and may guide them to the bone marrow (16). The noradrenaline secreted by the sympathetic nerve regulates the oscillatory CXCL12 expression in the bone marrow and elicits the egress of hematopoietic stem cells in a circadian manner (4). In addition, corticosterone oscillations regulate rhythmic murine CXCL12 and CXCR4 expression by bone marrow stromal progenitors (17). These observations demonstrate that systemic circadian signals can drive the oscillatory expression of pro-migratory factors.

The mechanism behind how the systemic circadian signals regulating the expression of pro-migratory molecules through the Bmal1 dominated molecular clock remains elusive. With a humanized mouse model, Zhao et al. have proved that the inner circadian environmental change is connected with

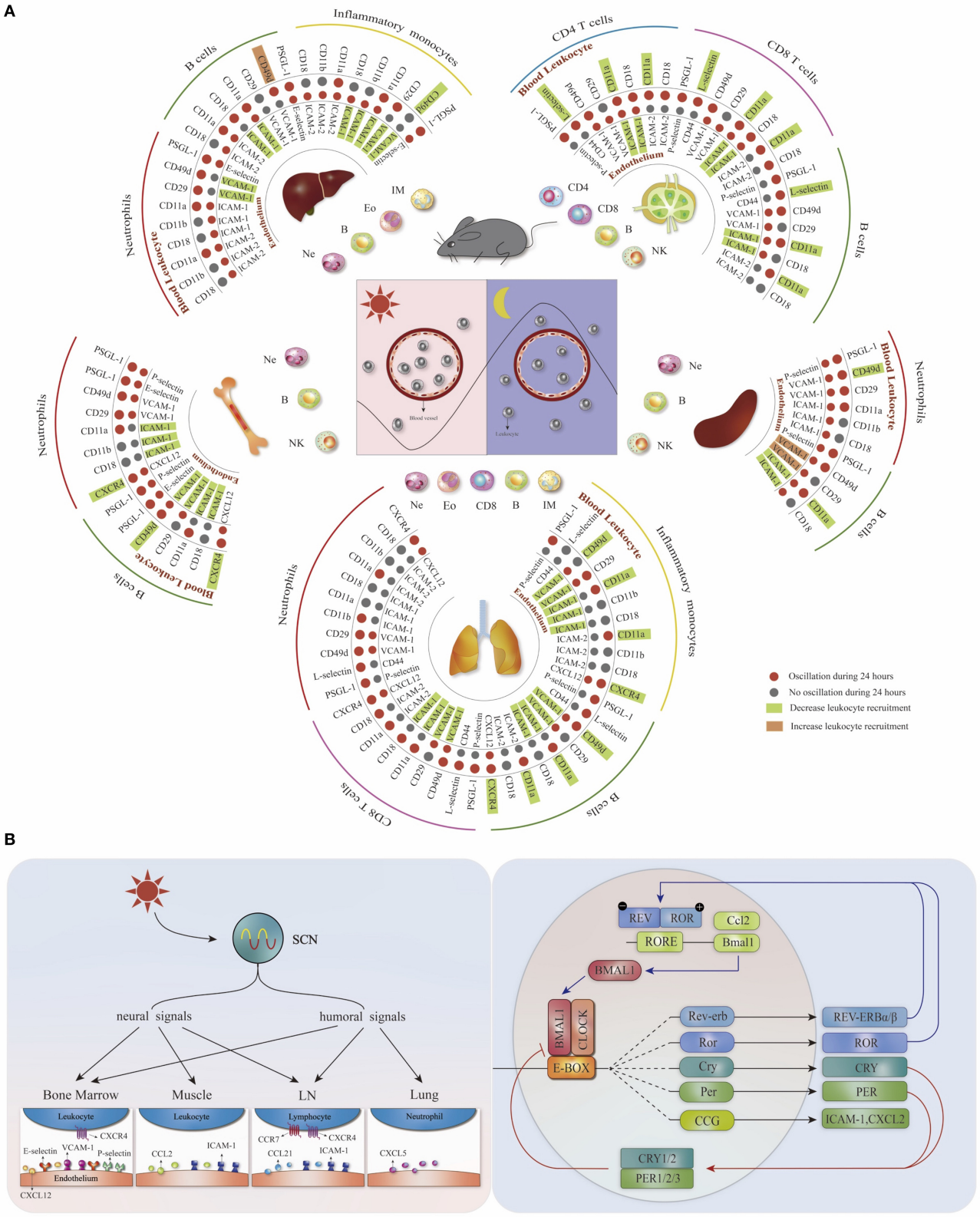

FIGURE 1 | The homing code and the mechanisms for regulating tissue- and leukocyte-specific homing. **(A)** Distinct leukocyte subsets recruited rhythmically (more in the evening) to different organs based on the tissue- and leukocyte- specific oscillatory expression (red dots) of pro-migratory molecules. Targeting different

(Continued)

FIGURE 1 | pro-migratory molecules can decrease (green square) or increase (orange square) the homing of specific leukocyte subsets, depending on the cell type as well as homing location. Modified, with permission, from He et al. (3). **(B)** The circadian system regulates the oscillatory expression of pro-migratory molecules through the systemic circadian signals as well as the cell-intrinsic molecular clock.

the cell molecule clock through the p38 mitogen-activated protein kinases/mitogen-activated 2 (MAPK/MK2)-ROS-HIF-1a-ARNTL1 pathway, which results in an opposite ROS level and inverse oscillation trend of CXCR4 expression in human and mouse leukocytes (18). Rhythmic glucocorticoids regulate the circadian expression of CXCL5 by mouse lung epithelial club (Clara) cells. Genetic ablation of Bmal1 in bronchiolar cells disrupts the CXCL5 rhythms despite persistent oscillatory glucocorticoid levels (19). Collectively, these studies suggest that the clock genes link systemic circadian signals with the oscillatory expression of pro-migratory molecules.

Together, these findings indicate that the circadian system regulates the oscillatory expression of pro-migratory molecules, thus influencing the homing process. Both the cell autonomous clock and the rhythmic microenvironment play an important part in the rhythmic homing of leukocytes into various organs (summarized in **Figure 1B**).

TISSUE-SPECIFIC HOMING OF LEUKOCYTE SUBSETS AND THE TIME-DEPENDENT EFFECTS

Leukocyte subsets migrate to different tissues with the help of the ligand–receptor interaction between the leukocytes and the endothelium. In this part, we summarize the driving molecules for the rhythmic homing of leukocyte subsets to specific tissue/organs and the functional role behind the rhythmic homing behavior (**Table 1**).

Lymph Node

The lymph node(LN) is mainly composed of CD4 T cells, CD8 T cells, and B cells, which are also the major leukocyte populations that home to the mouse lymph node (3). The lymphocytes accumulate in the mouse lymph node at the beginning of the night due to increased homing and reduced migration at this time point (20), which is governed by the dynamic expression of pro-migratory molecules including chemokine receptor CCR7, ICAM-1, and CCL21 on the high endothelial cell venules (3, 20). T-cell specific (*CD4-cre*) *Bmal1* knockout diminishes the rhythmic expression of CCR7 and thus ablates the rhythmic homing process of T cells (20), suggesting that the rhythmic recruitment of lymphocytes to LN is determined by the oscillatory expression of specific pro-migratory molecules mediated by the circadian clock. In addition, the sympathetic nerve regulates the rhythmic homing of lymphocytes to LN through β_2 adrenergic receptors (29), but it is unclear what pro-migratory molecules are controlled by the neural signaling in this process. The glucocorticoid receptor signaling elevates CXCR4 expression, which redistribute T cells between lymphoid organs and blood (21). These findings demonstrate that the rhythmic lymphocyte recruitment to LN is controlled by multiple circadian factors, including the molecule clock as well as the neural and humoral signals.

This diurnal oscillation of leukocyte number contributes to the time-dependent humoral immune response. Immunization of mice during the period of lymphocyte accumulation in LNs increases antibody titers (29). The severity of the experimental autoimmune encephalomyelitis mouse model is dependent on the time point of immune stimulation, and *CD4-cre Bmal1* knockout mice lose this time-dependent difference (20), suggesting that T cell rhythmic migration affects the time-dependent immune reaction. The influence of the LN rhythms is further demonstrated by immune response to pathogens. Mice infected with influenza A virus at ZT8 (ZT, *zeitgeber time*, ZT0 refers to light onset) can lead to stronger extent of pulmonary $CD8^{+}IFN\text{-}\gamma^{+}$ T-cell infiltration than at ZT20, 8 days post infection (20). Together, these data strongly indicate that the adaptive immune responses follow a circadian rhythm according to the LN number change.

In addition to lymphocytes, NK cells also exhibited a strong homing rhythmicity to the LN (3). Homed NK cells reside in the paracortex and the medulla of LN where they can be in contact with dendritic cells (DC). In addition, NK cells regulate colocalized T-cell responses in L. major infections through secreting INF-γ (30), but whether this NK–DC–T-cell interaction contributes to the time-dependent immune function needs to be further explored.

Bone Marrow

The bone marrow produces and releases leukocytes for the blood replenishment in a rhythmic manner, which is entrained by the sympathetic nerves (4). In addition, it's also an important site for mouse leukocyte rhythmic homing, including neutrophils, B cells, and NK cells (3, 22). The homing of mouse neutrophils to the bone marrow is dependent on CXCR4, ICAM-1, and L-selectin (3). Due to a short life span, the aged mouse neutrophils that shed L-selectin and exhibit high CXCR4 expression are dynamically eliminated from the circulation, which can clear the aged neutrophils and keep the immune homeostasis (22). Bone marrow macrophages engulf aged neutrophils and modulate the niche environment, resulting in the releasing of progenitor cells (22). Therefore, the rhythmic neutrophil homing links the blood environmental change and the bone marrow regeneration capacity.

In addition to neutrophils, B cells and NK cells also migrate into the mouse bone marrow in a circadian manner. B-cell homing to the bone marrow depends on VCAM-1, ICAM-1, CXCR4, and CD49d (3). The homed B cells reside in the perisinusoidal niche of the bone marrow and can freely recirculate and respond to blood-borne microbes, which extends the function of bone marrow as a secondary lymphoid organ (31). NK cells home to the bone marrow during viral infection for

TABLE 1 | Tissue specific leukocyte rhythmic homing and their effects.

Organ	Rhythmic homing cells	Driving molecules		Conditions	Effects	Reference
		Leukocyte	Vascular bed			
Lymph node	CD4 T cells, CD8 T cells	CCR7	CCL21	Steady state /EAE/Helicobacter pylori/influenza A virus	Heightened acquired immune function when stimulus occurred while lymphocytes accumulated in the lymph nodes	(20)
	CD4 T cells, CD8 T cells	CXCR4		Steady state	More rapid proliferation and efficient migration of lymph node T cells at night	(21)
Bone marrow	Neutrophils	CXCR4	CXCL12	Steady state	Neutrophil clearance modulates the hematopoietic niche, which contributes to the rhythmic egress of hematopoietic progenitors	(22)
Lung	Neutrophils			Steady state	Neutrophil aging and apoptosis	(3, 23)
			CXCL5	Inflammation	Time of day variation in the pulmonary inflammation and responses to bacterial infection	(24)
	B cells	CXCR4, CD11a, and CD49d	VCAM-1, ICAM-1	Steady state	Neutrophil aging and apoptosis	(3, 23)
Heart	Neutrophils	CXCR2	VCAM-1, ICAM-1, CXCL1, CXCL2, CXCL5, CCL3, and CCL5	Myocardial infarction	MI at ZT13 induces enhanced neutrophil infiltration and leads to poor prognosis	(25)
	Monocytes	CCR2	CCL2	Myocardial infarction	MI at ZT13 induces enhanced monocytes infiltration	(26)
Vessel	Neutrophils and monocytes	CCR2	CCL2	Atherosclerosis	Timed regime of blocking CCR2 during the activity phase inhibits atherosclerosis	(27)
	Neutrophils	CD11a, CD11b, CCR2 (artery) CD11a, CD11b, CCR2, CXCR2 (vein)	ICAM-1, VCAM-1	TNF-α induced acute inflammation	Time shifted leukocyte recruitment between artery and vein results in different thrombus formation time	(28)

the preservation of their ability to respond to subsequent viral challenge (22). These studies demonstrated that the recruitment of B cells and NK cells to the bone marrow is closely related to the immune responses. Therefore, the rhythmic migration behavior of those cells may influence the immune function in a time-dependent way.

Lung

The lung vasculature has a continuous and non-fenestrated endothelium to facilitate gas exchange and perform barrier functions (32). Under this special morphology, leukocytes recruit to the lung by firm attachment to the vessel wall, temporarily sequestered from the circulation blood, making the lung a reservoir of leukocytes (33).

In the steady state, mouse neutrophils home to the lung in a rhythmic manner, with more neutrophils attached to the vasculature at night (3, 34). Unlike the lymph node, spleen, and bone marrow, the homing of neutrophils to the lung is L-selectin independent (3), and some studies have suggested that spatial constrains seem to dominate neutrophil retention in lung capillaries (35). The diurnal infiltration of neutrophils to the lung maintains the rhythmic microenvironment of this organ, which controls the melanoma metastasis rhythm. Neutrophil-specific (*Lyz2-cre*) Bmal1 deletion abolishes this rhythmic recruitment of neutrophils as well as the melanoma metastasis rhythm (34), suggesting that the diurnal microenvironment of the lung is regulated by rhythmic neutrophil infiltrations and can change the susceptibility to diseases. For neutrophils, the lung provides a niche for the recruited neutrophils to encounter with B cells, which transfer MHCII to neutrophils and induce neutrophil aging (23). These data highlight that the rhythmic leukocyte homing is crucial for both the leukocyte physiology and the tissue circadian environment.

The homing of mouse neutrophils to the lung is also rhythmic in LPS-triggered pulmonary inflammation with more neutrophil infiltration in the daytime, which is induced by rhythmic expression of CXCL5 secreted by lung Clara cell (24). Although Clara cells have been shown to be under direct control of the glucocorticoid receptor-medicated repression, the neutrophilia persists with glucocorticoid receptor-depleted mice, suggesting other factors might be involved in neutrophil rhythmic migration in the inflammatory scenario (19). These data suggest that the

circadian control of pro-migratory factor is interplayed between multiple factors.

Besides neutrophils, mouse B cells, CD4 T cells, and inflammatory monocytes also recruit rhythmically to the lung. Blocking VCAM-1, ICAM-1, CD11a, or CD49d decreases the homing of these two cells (3). Future studies are needed to investigate the functions of the rhythmic homing of B cell and CD4 T cells to the lung and the interaction between different leukocyte subsets during their migration.

Heart and Vessel

The acute ischemic vascular events exhibit strong time-of-day dependency. Myocardial infarction (MI) occurs predominately in the morning in humans and is associated with unfavorable outcomes (36–38). This finding is well-reproduced in mouse MI model by permanent ligation of the left anterior descending coronary artery. Mice subjected to the MI model at ZT13 result in higher cardiac infarct size with more infiltration of neutrophils and monocytes compared to ZT5 (25, 26). At steady state, neutrophils and monocytes home rhythmically to the heart with the highest infiltration occurred at ZT13 during 24 h, and this time-dependent difference is greatly enhanced in MI situations depending on higher expression of CXCR2 and CCR2 on neutrophil and monocytes, respectively, together with corresponding adhesion molecules and chemokines on the endothelium (25, 26). Targeting CCR2 significantly inhibits monocyte infiltration at ZT13 but not ZT5 in the case of MI (26). Inhibiting the chemokine receptor CXCR2 or using neutrophil-specific CXCR2 knockout remarkably reduces the infarct size and preserves cardiac function after the occurrence of MI (25). These data suggest that the chrono-pharmacological treatment targeting the rhythmic migration behavior of the leukocytes can provide a favorable treatment outcome. The influence of the circadian system on MI is further demonstrated by the homozygous clock mutant mice($Clock^{delta19}$), which leads to more infiltrations of neutrophils and macrophages to infarcted myocardium, and worse cardiac structure, suggesting that abolishment of circadian rhythm can adversely affected the cardiac remodeling (39). REV-ERB agonist SR9009 provides beneficial effects in long-term cardiac repair for mice post-myocardial ischemia reperfusion (40). Collectively, the circadian rhythm modulating inflammatory responses is crucial for the myocardia recovery and prognosis.

The atherosclerosis is a chronic inflammation of the arterial wall, which greatly involves leukocyte recruitment and can lead to acute cardiovascular events (41). Myeloid cells recruit to the atherosclerotic lesions in a circadian fashion with a peak during the early activity phase, which is driven by rhythmic level of myeloid cell-derived CCL2 immobilized on the endothelial cells. CCR2 on myeloid cells shows in phase oscillation with CCL2. Together, CCL2-CCR2 triggers rhythmic adhesion of myeloid cells. Myeloid *Bmal1*-specific knockout mice (*Lyz2-cre*: *Arntl flox*) lose rhythmic expression of CCL2 and oscillation of myeloid cell adhesion, suggesting that the circadian expression of CCL2 mediated by BMAL1 is responsible for the rhythmic homing of myeloid cells. The rhythmic behavior of myeloid cells provides a timed treatment strategy for CCR2 neutralization with antagonist RS102895, which efficiently reduced atherosclerosis with a short-time administration at ZT17 without disturbing microvascular recruitment (27), supporting the concept that the time and site-specific leukocyte recruitment pattern provides us with a beneficial manner to regulate the leukocyte migration with time-tailored therapy.

Besides atherosclerosis, thrombotic vascular occlusion also exhibits strong circadian rhythm, which is strongly dependent on the circadian clock control (42). A recent study reveals that myeloid cells recruit to arteries and veins in a phased delayed rhythmic manner in the TNF-α induced acute inflammation model. This time shift of myeloid cell adhesion is regulated by a vessel type specific oscillatory pattern in the expression of pro-migratory molecules, driven by the intrinsic autonomous clock as well as local sympathetic innervation. The distinct leukocyte adhesion patterns in the vein and artery result in a different acute thrombus formation time in the vein (at ZT2) and artery (at ZT8) in a phototoxicity-induced thrombus model (28). The experiments described above highlight the different circadian rhythmicity between veins and arteries.

CONCLUSION

In summary, leukocyte subsets home to different tissue/organs governed by the interaction of distinct pro-migratory molecules expressed on endothelial cells and leukocytes. The circadian system regulates the time-dependent expression profiles of the pro-migratory molecules by multiple factors that are only partially known. Future studies are needed to investigate how the synchronization signals interplay with the peripheral molecule clock to regulate the expression of pro-migratory molecules. The leukocyte rhythmic homing plays important functional roles in various aspects, including immune responses, the replenishment of the blood, as well as the tumor metastasis rhythms. Targeting specific leukocyte rhythmic homing in a time-tailored way has proven to be a beneficial method in some pathological conditions. To create new possibilities for the chronotherapies for human patients, more experiments with mechanistic insights into the circadian leukocyte migration are needed. In addition, the functional roles of leukocyte rhythmic trafficking should be further explored.

AUTHOR CONTRIBUTIONS

YY and SW drafted the article and designed the figure. WH devised the structure of the review and wrote the manuscript. WL aided in revising the manuscript. All authors give approval for publication.

FUNDING

This work was supported by a welcome starting grant of the Xinqiao Hospital of Army Medical University.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 03 April 2020
doi: 10.3389/fimmu.2020.00576

Circadian Features of Neutrophil Biology

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Rhythms in immunity manifest in multiple ways, but perhaps most prominently by the recurrent onset of inflammation at specific times of day. These patterns are of importance to understand human disease and are caused, in many instances, by the action of neutrophils, a myeloid leukocyte with striking circadian features. The neutrophil's short life, marked diurnal variations in number, and changes in phenotype while in the circulation, help explain the temporal features of inflammatory disease but also uncover core features of neutrophil physiology. Here, we summarize well-established concepts and introduce recent discoveries in the biology of these cells as they relate to circadian rhythms. We highlight that although the circadian features of neutrophils are better known and relevant to understand disease, they may also influence important aspects of organ function even in the steady-state. Finally, we discuss the possibility of targeting these temporal features of neutrophils for therapeutic benefit.

Keywords: neutrophil, circadian inflammation, chronotherapy, molecular clock, oscillatory signals

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Krisztina Káldi, Semmelweis University, Hungary
Sara M. Rankin, Imperial College London, United Kingdom

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *25 November 2019*
Accepted: *12 March 2020*
Published: *03 April 2020*

Citation:
Aroca-Crevillén A, Adrover JM and Hidalgo A (2020) Circadian Features of Neutrophil Biology. Front. Immunol. 11:576. doi: 10.3389/fimmu.2020.00576

GENERAL FEATURES OF CIRCADIAN IMMUNITY

The rotational period of the Earth creates variations in sunlight exposure and provides regular diurnal cues to organisms (1). Sensing of these cues synchronizes organismal physiology and behavior with external conditions (2), thus providing an evolutionary advantage by allowing organisms to anticipate and adapt to a changing environment (1). These cyclic biological changes create circadian rhythms which are endogenous, self-maintained oscillations that display a periodicity of ∼24 h (3). These rhythms are entrainable by periodic changes in environmental cues, such as light or food (4, 5). Central and peripheral mechanisms regulating circadian oscillations in organisms and in cells have been reviewed extensively, including in the immune system, and will not be further reviewed here (6, 7).

Circadian rhythms are present in many cellular and humoral components of the immune system. Granulocytes and monocytes exhibit circadian oscillations in their numbers in blood, both in humans (8) and mice (9), and these oscillations are also robust in T- and B-lymphocytes (10–12). Circadian variations in clock gene expression have also been reported in many types of immune cells, including monocytes (13, 14), macrophages (15, 16), neutrophils (17, 18), dendritic cells (12), or lymphocytes (10, 12). This suggested the presence of functional, intrinsic clockworks in immune cells, and recent studies have demonstrated that many immune processes are under direct circadian control. For example, rhythmic leukocyte recruitment is regulated by circadian expression of pro-migratory factors within endothelial cells (9), circadian trafficking of lymphocytes through lymph nodes is controlled by Bmal1-dependent expression of the receptor CCR7 (19), and the response of phagocytes to *Leishmania* infection is abolished in mice lacking the molecular clock in innate immune

cells (20). Overall, these and many other observations have expanded the ascribed role of circadian clockworks and oscillatory signals within the microenvironment in the control of immune cell trafficking and host-pathogen interactions. Consistent with variations in the immune cell number and function, inflammatory diseases display circadian manifestations. Prominent among these are those that affect the cardiovascular system, with acute vascular events displaying rhythmic patterns in both onset and severity. For instance, myocardial infarction in mice and humans shows circadian variations in both onset (for humans) and infarct size depending on the time of day, with evidence supporting changes in leukocyte infiltration rates into the myocardium (17, 21–23). Occurrence of ischemic stroke also has a peak of incidence in the morning (24), in coincidence with higher atherosclerotic plaque rupture at this time (25). Likewise, certain autoimmune disorders such as rheumatoid arthritis exhibit daily variations in joint inflammation with stiffness and pro-inflammatory cytokines peaking in the morning (26, 27). Finally, sepsis modeled by caecal ligation or lipopolysaccharide (LPS) injection also shows circadian variations, with increased severity during the night in mice (9, 28). Below, we focus our discussion on the circadian properties of neutrophils, a type of leukocyte whose short life cycle appears to have adapted optimally and in multiple ways to the circadian rhythms of mammals (29, 30).

CIRCADIAN FEATURES OF NEUTROPHILS IN THE BONE MARROW

Neutrophils are mainly produced within the bone marrow (BM) through a process known as *granulopoiesis*. A complex interplay between the transcription factors PU.1, enhancer-binding proteins (C/EBPs), Gfi-1 and GATA-1 determines the commitment of immature progenitors to the myeloid-lineage [reviewed in (31)]. From this point on, C/EBPα induces the expression of the granulocyte colony stimulating factor receptor (G-CSFR/CSF3R), which allows signals delivered by the cytokine granulocyte-colony stimulating factor (G-CSF/CSF3) to promote granulopoiesis (32). Insights based on single cell analyses have additionally defined committed neutrophil precursors reliant on the transcription factor C/EBPε (33, 34) **(Figure 1)**. Recent reviews have already described basic aspects of granulopoiesis, including the different stages of maturation both under homeostasis or emergency (30, 35, 36), and will not be discussed here further. A more detailed characterization of the signals that control these developmental stages will be needed to determine the possible existence of a circadian component that boosts granulopoiesis at certain times of the day.

The BM maintains a neutrophil pool ready to be released under homeostatic and stress conditions. Given the toxic potential of neutrophils, granulopoiesis, and subsequent release must be tightly regulated to balance their numbers in the circulation. This is achieved by massive daily production [up to 2×10^{11} cells per day in humans (37)] and temporally-gated release into blood (38). Circulating neutrophils ultimately infiltrate tissues after only 6–10 h in the circulation, which makes them one of the shortest-lived cells in our bodies. It is noteworthy that the percentage of mature neutrophils in human and mouse peripheral blood is between 50–70 and 10–25%, respectively (30). These differences can make difficult to accurately translate findings across species. Under homeostatic conditions immature neutrophils are largely absent from the circulation in both human and mice (33). However, under inflammatory conditions the number of immature neutrophils increases in blood, as shown by the increase of the CD101-negative population in the circulation of tumor-bearing mice (33) or the release of CD16-dim neutrophils from the BM in a model of human endotoxemia (39).

Oscillatory signals within the BM are believed to play an important role in both release and clearance. Studies in mice have shown that the chemokine CXCL12, acting through its receptor CXCR4, provides a key retention signal for neutrophils (and other cells) within the BM (40, 41). Importantly, regulation of CXCL12 levels in the mouse BM appears to be controlled by neural signals. Sympathetic nerves that innervate the BM deliver diurnal adrenergic signals to stromal cells through β3-adrenergic receptors, which inhibit CXCL12 expression and generate oscillatory expression of the chemokine (42). Cholinergic signals from the parasympathetic nervous system (PNS), in turn, have been shown to inhibit adrenergic activity of the murine SNS at night (43), altogether establishing tight temporal patterns in the BM. In mice, downregulation of CXCL12 at daytime drives the circadian egress of HSCs (42), and the release of neutrophils at this same time also coincides with decreased CXCL12 (40) **(Figure 1)**. Several lines of evidence suggest that timed release through CXCL12 underlies the circadian variations of neutrophil numbers in blood: first, administration of a CXCR4 antagonist mobilizes neutrophils from the BM in both mice (44) and humans (45, 46), although another study found that the antagonist mobilized neutrophils from the lungs of both mice and macaques (47); second, genetic deletion of *Cxcr*4 only in myeloid cells results in massive neutrophilia in blood (41), and interestingly also results in blunted oscillations of neutrophils in blood (17). In addition to the CXCL12/CXCR4 axis, CXCR2 also plays an important role in neutrophil trafficking (44, 48) as its absence leads to neutrophil retention in the BM, and partly counteracts CXCR4 to regulate the egress of neutrophils (49). Interestingly, the CXCR2 ligands CXCL1 and CXCL2 are constitutively expressed by BM endothelial cells and osteoblasts (49) **(Figure 1)**, and their expression can be also enhanced by external stimuli, including G-CSF (49) or thrombopoietin (50), thus contributing to neutrophil mobilization. Together, these results highlight the tight regulation of cues driving neutrophil egress, all of which are likely subjected to circadian control in a manner similar to CXCL12, but this needs to be explored further.

After circulating for several hours, mature neutrophils are ultimately cleared in different tissues (see discussion below). Among these, it is interesting that the BM is one of the major clearance sites for neutrophils (51, 52) in a process also controlled by the CXCL12/CXCR4 pathway in both humans and mice (41, 44, 48, 53). Adoptive transfer experiments showed that the population of neutrophils that preferentially homes to the BM expresses higher levels of CXCR4, which agrees with the

notion that CXCR4^{HI} aged neutrophils (those that have remained longer in the circulation; see below) gain tropism for the BM as part of their programmed lifecycle (44). There are, however, contradictory observations as homing experiments indicated that CXCR4^{HI} aged neutrophils have a similar ability to infiltrate the BM as CXCR4^{LO} neutrophils (53). In addition to neutrophil-intrinsic changes, other studies have shown the importance of environmental factors in modulating the circadian recruitment of leukocytes into tissues under homeostatic conditions. For example, expression of P- and E-selectins as well as VCAM-1, controlled by the SNS, oscillate in a circadian fashion in the BM vasculature, and favor the recruitment of leukocytes at night in mice (9).

Adding to these circadian aspects of neutrophil trafficking to and from the BM, clearance of neutrophils in this organ has been shown to regulate the hematopoietic niche. Studies in mice showed that aged neutrophils entering the BM are engulfed by BM macrophages (51). Through a process dependent on the LXR nuclear receptors, these cells trigger reductions in CXCL12 expression and alter the cellular composition of the hematopoietic niche, altogether promoting the egress of hematopoietic stem cells (HSCs) into the circulation (53) (**Figure 1**). The return of neutrophils to the BM at the end of the resting phase (in mice; between ZT5 and ZT13) is promoted by SNS-dependent, circadian regulation of CXCL12 and other molecules required for HSC homing (9). These highly-regulated processes contribute to control neutrophil numbers and the properties of the BM throughout the day.

CLOCK-DRIVEN PHYSIOLOGY OF THE CIRCULATING NEUTROPHIL

The remarkably short lifespan of neutrophils in blood implies that many resources must be employed in their production (54). This feature likely relates to evolutionary trade-offs for a cell that is key to immune defense, but is also highly cytotoxic and can incite vascular inflammation (55). A consequence of this rapid turnover is that neutrophil numbers follow strong circadian changes in blood. Remarkably, these circadian oscillations also affect the phenotype of circulating neutrophils, a property referred to as neutrophil *aging* (29). Most data on neutrophil aging classically derived from *in vitro* studies revealing, for example, increased surface levels of CXCR4 (44), and decrease of CXCR2 (49) or L-selectin (56). The physiological impact of this circadianally-regulated phenomenon, and the underlying molecular mechanisms, have remained unclear until recently.

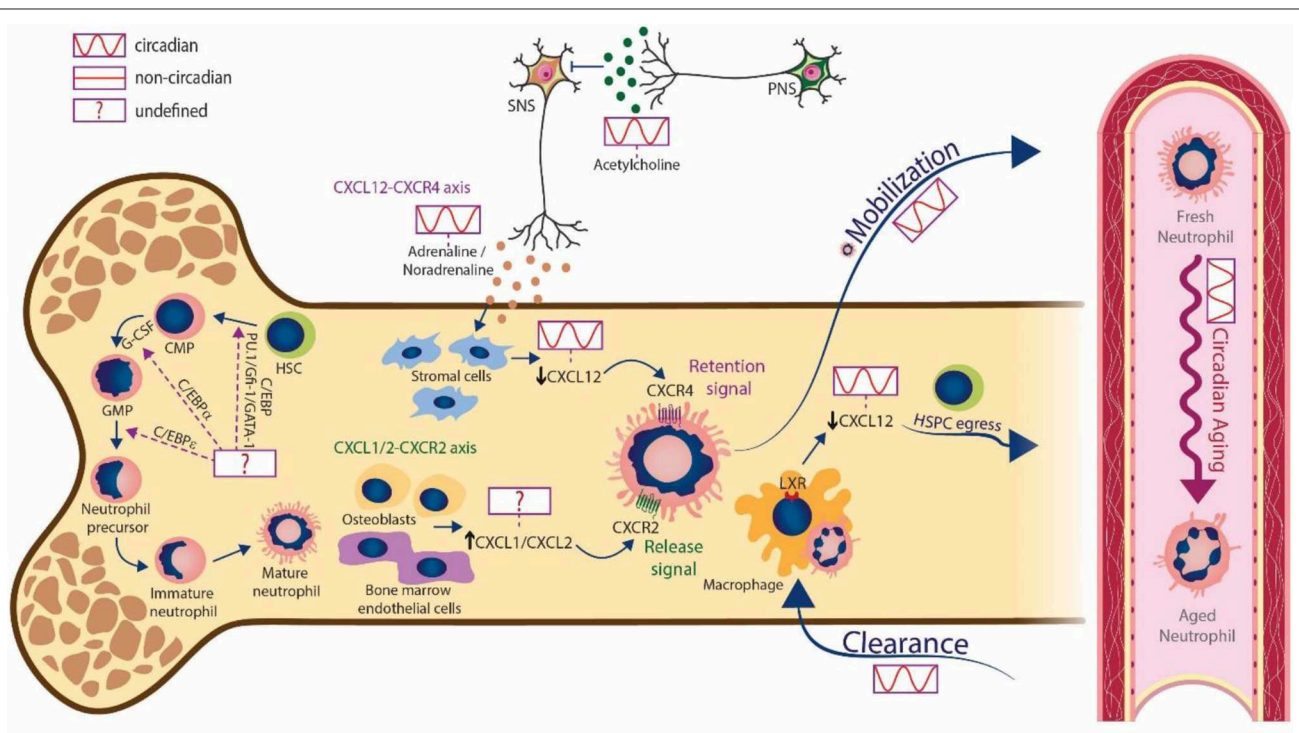

FIGURE 1 | Circadian regulation of neutrophils in bone marrow and blood. Mature neutrophils are produced in the bone marrow during granulopoiesis. The transcription factors PU.1, Gfi-1, GATA-1 and different enhancer-binding proteins (C/EBPs) are involved in this process, but the existence of oscillatory changes in their expression is unknown. The sympathetic nervous system releases cues (adrenaline and noradrenaline) that act on stromal cells to generate circadian changes in CXCL12 levels. This ultimately decreases the expression of CXCL12 and promotes the circadian release of neutrophils into the bloodstream. In turn, the parasympathetic nervous system suppresses the activity of the SNS trough cholinergic signals (acetylcholine). In addition to the CXCR4/CXCL12 axis, signaling through CXCR2 by the chemokines CXCL1 and CXCL2 produced by osteoblasts and bone marrow endothelial cells, also mediates neutrophil egress, however the circadian regulation of this axis needs further investigation. Neutrophils undergo aging in circulation following circadian patterns and are finally cleared into the bone marrow and other tissues. The engulfment of aged neutrophils by macrophages activates LXR signaling, which in turn blunts expression of CXCL12 to promote the circadian egress of hematopoietic stem and progenitor cells (HSPCs). Note that cell morphologies are characteristic from mice.

Using a model of neutrophil transfer into antibiotic-treated mice, a study proposed that neutrophil aging is controlled by extrinsic, microbiota-derived and TLR4-dependent signals (57). A caveat of these studies was that no links were established with actual circadian timing, thus making the temporal relevance of the findings unclear. More recently, another study reported that neutrophil aging was controlled cell-intrinsically by the core clock gene *Arntl* (encoding Bmal1), was entrained by light, and was dependent on antagonistic CXCR2 and CXCR4 signaling (17). Interestingly, neutrophil-intrinsic Bmal1 regulated the circadian expression of CXCL2, a chemokine that signaled in an autocrine fashion through CXCR2 to promote the transcriptional and phenotypic changes associated with neutrophil aging. In the proposed model, CXCR4 counteracted signaling through CXCR2, thereby blocking this program (17). This is consistent with studies in humans showing circadian variations in plasma levels of CXCL12 (the ligand for CXCR4) in antiphase with the aging phenotype (17, 18), although it is noteworthy that these studies found that the levels of BMAL1 are very low in human peripheral neutrophils. Thus, circadian neutrophil aging appears to be cell-intrinsically regulated by the molecular clock, and extrinsically through CXCR4 signaling. Because the same chemokine receptors that control the egress of neutrophils from the BM (49) also control aging, we propose that CXCR4 signaling temporally coordinates the release of neutrophils into blood with the onset of aging only in peripheral blood, possibly protecting the BM from the potentially toxic activity of activated neutrophils. Since CXCR4/CXCL12 signaling is controlled circadianally by sympathetic signaling under steady-state conditions (9, 58) it will be interesting to explore how chronic or acute inflammation alter the circadian properties of neutrophils.

An intriguing finding from these studies was that aged neutrophils are preferentially cleared out from the circulation into healthy tissues under steady-state conditions (17), whereas non-aged cells ("fresh" neutrophils) are preferentially recruited to inflammatory sites. This was explained by the progressive loss of microvilli needed for efficient rolling as neutrophils aged over time (17). Further, expression of CXCR2 is reduced in aged neutrophils and we have recently shown that these cells feature reduced granule content and NET-forming capacity relative to fresh neutrophils (59), altogether suggesting blunted inflammatory properties for aged neutrophils. However, these blunted inflammatory properties are in apparent contradiction with reports showing elevated inflammogenic properties of aged neutrophils in mice (57), as well as increased adhesion, ROS production and phagocytic capacity in human aged neutrophils (18). The reason for these discrepancies deserves further investigation.

The pathophysiological consequence of this clock-controlled behavior of circulating neutrophils has been put in manifest in the context of infection and vascular inflammation. For example, enhanced seeding of tissues like the kidney by (aged) neutrophils at night protected from fungal infection. In a model of *Candida albicans* infection, pathogen clearance was superior at night, a time when neutrophils had already entered the tissue, and exaggerated neutrophil aging by deletion of CXCR4 markedly protected against infection. In contrast, deletion of *Arntl* rendered mice more susceptible to infection at night (17). Similar outcomes were found in the context of sterile inflammation, as the circadian differences in ischemic stroke or myocardial infarction were also sensitive to the deletion of Bmal1 in neutrophils (17). An interesting conclusion from these experiments is that neutrophil numbers in blood, which have been correlated with vascular disease and used for prognosis in the clinics (60), may not be the key factor in disease outcome while, at least in mice, the aging phenotype of the cells is a better predictor of the immune response.

NEUTROPHIL IN TISSUES AND CIRCADIAN PATHOPHYSIOLOGY

Commonly believed to be eliminated only in the BM, spleen and liver (61), neutrophils have now been shown to infiltrate many other tissues in the steady-state (at least in mice), including the intestine, lung, white-adipose tissue (WAT), skin, skeletal muscle, lymph nodes, kidneys and heart (52). Notably, infiltration of neutrophils into most naïve tissues follows circadian patterns with a peak at night, with exceptions in the intestine, liver and WAT in which no circadian oscillations were detected (52) **(Figure 2)**. Remarkably, the function of neutrophils in most of these tissues remains virtually unexplored.

An outstanding question is whether tissue-infiltrating neutrophils organize in specific areas that enables particular functions in each tissue. In the intestine, for example, neutrophils distribute in clusters around isolated lymphoid follicles, and are surrounded by CD169+ macrophages (52). In this case, the proximity to IL-23-producing cells predicted regulation of the levels of this cytokine and downstream production of G-CSF, an important mobilizing cytokine. Indeed, we identified a role for gut-infiltrating neutrophils in regulating systemic G-CSF levels and subsequent mobilization of hematopoietic stem and progenitor cells (HSPCs) from the BM (52) **(Figure 2)**. Intriguingly, this regulatory role appeared to be unrelated of the circadian release of HSPC in blood (42, 53). Whether neutrophils in the gut coordinate with other oscillatory signals within this environment, such as the intestinal microbiota (62), remains to be explored.

Contrary to the gut, neutrophil infiltration of the lungs follows tight circadian patterns (52). The need to defend against colonizing bacteria may explain the existence of a large marginated pool of neutrophils within the lung microcirculation, as shown both in human and mice (63). In mice, the lung has been proposed to be an "education" site for neutrophils incoming from injured tissues to promote their return to the BM (64). Interestingly, in the mouse lungs neutrophils were shown to entrain global circadian transcription that appeared to predispose to organ invasion by metastatic cells (52), thereby suggesting that diurnal neutrophil clearance in the lung may influence the temporal dynamics of patho-physiological processes. The mechanisms underlying diurnal regulation of circadian expression in this tissue, and whether this could be extended to other tissues, remains unknown. Reciprocal

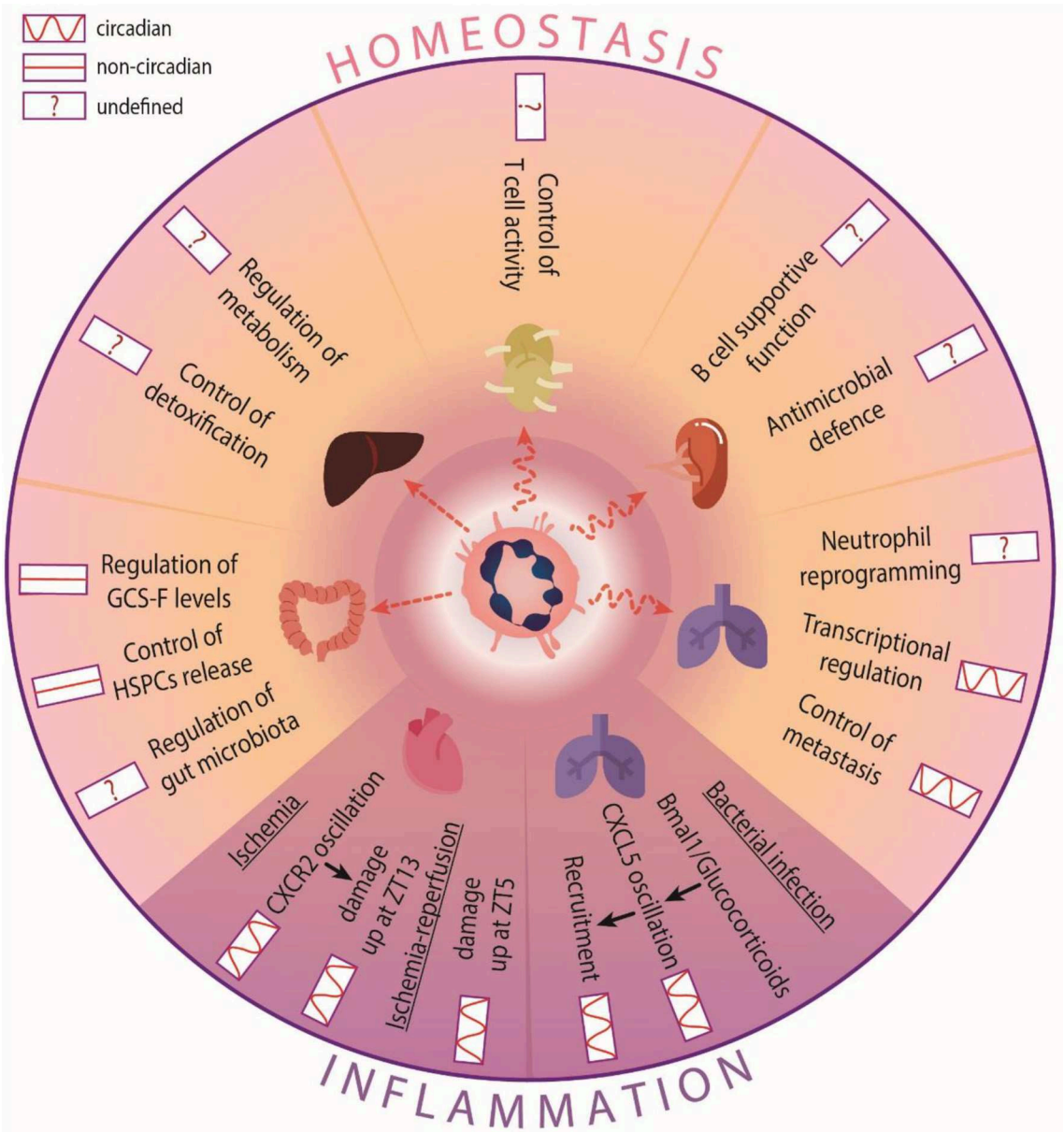

FIGURE 2 | Circadian functions of neutrophils in tissues. In homeostasis, neutrophil infiltration into most tissues is circadian, but not in the intestine or the liver. Neutrophils perform different functions in a circadian-independent manner: In the intestine, they control G-CSF production to mobilize HSC. In other tissues they regulate circadian processes, such as transcriptional programs and tumor invasion in the lung. The circadian influence in other tissues is not well-defined. For example, metabolism or detoxification control in the liver, gut microbiota regulation in the intestine, or their own re-education and immune defense in the spleen or lymph nodes, respectively. In inflammatory scenarios, neutrophil recruitment oscillates and influences disease outcome. During bacterial infection in the lung, bronchiolar cells modulate CXCL5 expression to control the oscillatory recruitment of neutrophils. In models of cardiac ischemia, increased infiltration into the heart accounts for exacerbated cardiac damage at different times depending on the type of injury performed.

regulation is also possible, and indeed extrinsic regulation of circadian recruitment of neutrophils during bacterial infection is potently mediated by bronchiolar cells, whose expression of attractant chemokine CXCL5 is regulated by glucocorticoids and Bmal1 in a circadian manner (65) **(Figure 2)**.

The liver is a preferential site for neutrophil elimination (30) (**Figure 2**). In this organ, many physiological functions such as energy metabolism or detoxification are under circadian control (66) and disruption of the hepatic clock promotes disease, including cirrhosis, hepatic steatosis and liver cancer (66), some of which have been shown to be regulated by neutrophils (67). Whether timed infiltration of neutrophils in this tissue influences these or other physiological liver functions remains to be explored.

In hematopoietic and lymphoid tissues other than the BM, such as the spleen, neutrophils have been reported to promote

maturation, differentiation, and antibody production of B cells (68). Neutrophils at different stages of maturation have also been postulated to perform antimicrobial functions in the spleen, and facilitate clearance of *Streptococcus pneumonia* (69). Recently, a study has shown that MHCII+ neutrophils in lymph nodes interact with dendritic cells and macrophages, presumably to modulate T cell activation **(Figure 2)**, with the caveat that this was largely shown *ex vivo* using bone marrow-derived cells (70). Given the rhythmic recruitment of neutrophils to both spleen and lymph nodes (52, 71), it is conceivable that certain aspects of adaptive immunity and antimicrobial properties of these organs are circadianally regulated by neutrophils, but this too needs further investigation.

A key question is what dictates the temporal pattern of neutrophil entry into the different tissues. A recent study found that cell-intrinsic signals regulated by Bmal1, as well as environmental oscillations of migratory factors orchestrate the rhythmic trafficking of neutrophils and other leukocytes to different tissues in both homeostatic and inflammatory conditions, in human and mice (71). Blockade experiments performed at ZT13, coinciding with neutrophil exit from the circulation, showed that CXCR4 and ICAM-1 in the BM, L-selectin in the lymph nodes and spleen, and VCAM-1 in the liver, are the main factors that guide neutrophil emigration to those tissues, with oscillations in both neutrophils or endothelial cells (71). These detailed studies raise the possibility of exploiting this circadian signature of migration for chronotherapy.

The rhythmic recruitment of neutrophils could be also responsible for the circadian manifestation of various inflammatory diseases. In a model of myocardial ischemia, the exacerbated infiltration of neutrophils at night (ZT13) accounted for increased cardiac damage at this time (22). In this case, the differential recruitment appeared to be CXCR2-dependent (22). This correlates with data showing increased CXCR2 expression on neutrophils at night (17). Interestingly, in the context of myocardial ischemia-reperfusion, infarct sizes were larger in the morning (17, 23), suggesting that the type of injury (ischemic or after reperfusion) follows distinct circadian patterns. Altogether, these studies uncovered the importance of circadian neutrophil infiltration across different tissues, with potential implications in the treatment of inflammatory disease **(Figure 2)**.

TARGETING THE CIRCADIAN PROPERTIES OF NEUTROPHILS FOR THERAPY

Several studies have demonstrated the importance of the (circadian) time parameter in clinical settings, which raised the possibility of using these temporal physiological features for therapeutic benefit (i.e., chronotherapy). Indeed, administration of drugs at specific times of day in diseases such as cancer or asthma, or the performance of surgical procedures at specific times, has often resulted in enhanced therapeutic success (7). These findings highlight the possibility of "personalizing" medicine at the temporal level.

The historical reticence to target neutrophils therapeutically is explained by their essential antimicrobial function. However, a wealth of studies in the past few years have identified heterogeneity among neutrophils, raising the possibility of targeting only specific, disease-causing subsets. In arthritic mice, G-CSF receptor blockade decreases disease progression by inhibiting neutrophil accumulation and local production of pro-inflammatory cytokines without affecting their defensive function (72). *In vivo* interference with the production of neutrophil extracellular traps (NETs) has been shown to be protective in systemic lupus erythematosus (SLE) (73) and transfusion-related acute lung injury (TRALI) (74), whereas the β1-adrenergic-receptor antagonist metoprolol decreases infarct size during AMI by interfering neutrophil recruitment and neutrophil-platelet interactions (75). These and other examples postulate the possibility to target neutrophils therapeutically, as reviewed recently (76, 77). Given the observation that the molecular clock influences the effector functions of neutrophils, an outstanding question is whether this "neutrophil clock" can be targeted to prevent inflammatory disease.

An extensive transcriptomic study focused on rhythmic gene expression in whole tissues revealed that many common anti-inflammatory drugs, which in turn have short half-lives, can be directed to circadian genes or their products, thus pointing out the potential therapeutic benefit of targeting clock genes and dosing clock-directed drugs at optimal times to improve their effectiveness (78). As an example, disruption of the circadian clockwork in macrophages eliminates the exacerbated endotoxin-induced cytokine response observed at night by suppressing the expression of the circadian repressor REV-ERBα (79). This is of importance since a synthetic REV-ERB ligand (GSK4112) was shown to attenuate cytokine production by macrophages (79), in what was one of the first proof of concept studies that targeted molecular clock proteins to modulate inflammation. This aligned with studies showing the beneficial effect of the REV-ERB agonist SR9009 in reducing atherosclerotic plaque size in LDL receptor-deficient mice (80). In addition, *in vitro* targeting of the repressor clock protein CRY with the activator KL001 also demonstrated anti-inflammatory effects in chronic arthritis (27). Despite the *in vitro* nature of these reports, clock-mediated therapy for immune-mediated diseases emerges as a valuable therapeutic tool, which we expect will be soon also exploited in neutrophils.

Although the full extent of the physiological consequences of circadian rhythms in neutrophils is still unclear, recent studies have suggested its therapeutic potential. For example, disruption of Bmal1 in club cells and adrenalectomy in the context of circadian recruitment of neutrophils to lungs after LPS revealed blunted glucocorticoid signaling, non-rhythmic expression of CXCL5, neutrophilia and antimicrobial responses (65). Other studies tested the possibility of targeting the circadian properties of neutrophils and monocytes in atherosclerosis, by showing arterial- and time-specific repression of leukocyte recruitment to plaques upon inhibition of CCR2 (81). Timed CCR2 blocking at nighttime (ZT17) decreased arterial myeloid cell recruitment and atherosclerotic lesion formation, whereas the neutralization at daytime (ZT5) had no effect, suggesting again the importance of

chrono-pharmacology-based approaches (81). Finally, targeting pro-migratory factors VCAM-1, ICAM-1 and CD49d during inflammatory challenge with LPS affected neutrophil trafficking and blunted inflammation (71). Overall, these findings have provided strong evidence that targeting circadian mechanisms specific to the immune system may have therapeutic value. Moving forward, we propose that complete characterization of the circadian features of neutrophils, including our recent identification of a cell-intrinsic circadian "timer" (17), will yield powerful new strategies to bring time, and immunity, on the patient's side.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing of this review. AA-C prepared figures. JA wrote a section of the review. AH coordinated the writing and edited the text and figures.

FUNDING

The project that gave rise to these results received the support of a fellowship from la Caixa Foundation (ID 100010434), fellowship code for AA-C was LCF/BQ/DR19/11740022. JA was supported by fellowship BES-2013-065550 from Ministerio de Ciencia, Innovacion y Universidades (MCIU). This work was supported in part by grant RTI2018-095497-B-I00 from MCIU, HR17_00527 from La Caixa Foundation, and TNE-18CVD04 from the Leducq Foundation to AH. The CNIC was supported by the MCIU and the Pro-CNIC Foundation, and was a Severo Ochoa Center of Excellence (MCIU award SEV-2015-0505).

ACKNOWLEDGMENTS

We are grateful to our funding sources.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 20 May 2020
doi: 10.3389/fimmu.2020.00956

The Autonomic Nervous System Pulls the Strings to Coordinate Circadian HSC Functions

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As for many other adult stem cells, the behavior of hematopoietic stem and progenitor cells (HSPCs) is subjected to circadian regulatory patterns. Multiple HSPC functions, such as proliferation, differentiation or trafficking exhibit time-dependent patterns that require a tight coordination to ensure daily blood cell production. The autonomic nervous system, together with circulating hormones, relay circadian signals from the central clock—the suprachiasmatic nucleus in the brain—to synchronize HSC niche physiology according to light/darkness cycles. Research over the last 20 years has revealed how specific neural signals modulate certain aspects of circadian HSC biology. However, only recently some studies have started to decipher the cellular and molecular mechanisms that orchestrate this complex regulation in a time-dependent fashion. Here we firstly review some of the recent key findings illustrating how different neural signals (catecholaminergic or cholinergic) regulate circadian HSC egress, homing, maintenance, proliferation, and differentiation. In particular, we highlight the critical role of different neurotransmitter receptors in the bone marrow microenvironment to channel these neural signals and regulate antagonistic processes according to circadian cues and organismal demands. Then, we discuss the potential biological meaning of HSC circadian regulation and its possible utility for clinical purposes. Finally, we offer our perspective on emerging concepts in HSC chronobiology.

Keywords: autonomic nervous system, circadian, hematopoietic (stem) cells, adrenergic, cholinergic

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Yuya Kunisaki, Kyushu University, Japan
Sandra Pinho, University of Illinois at Chicago, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *05 March 2020*
Accepted: *23 April 2020*
Published: *20 May 2020*

Citation:
García-García A and Méndez-Ferrer S (2020) The Autonomic Nervous System Pulls the Strings to Coordinate Circadian HSC Functions. Front. Immunol. 11:956. doi: 10.3389/fimmu.2020.00956

INTRODUCTION

Throughout evolution, most species have developed the capacity to adapt their behavior and physiology to the day-night oscillations derived from earth's 24 h rotation. In mammals, these so-called circadian functions are synchronized by light-darkness shifts, and influenced by other factors, such as alimentary habits. The circadian system has at least two different levels of complexity. Centrally, the suprachiasmatic nucleus, located in the hypothalamus, is the anatomical structure that orchestrates the organism's circadian activities. This master clock receives photic input from photosensitive neurons in the retina through the retino-hypothalamic tract and processes this information to synchronize the circadian rhythms in peripheral tissues. However, most cells display at least one intrinsic or internal clock that influences their proliferation, differentiation and

migration properties. This cellular clock is periodically reset by neuroendocrine signals emanating from the suprachiasmatic nucleus, which resets or synchronizes the peripheral oscillators throughout the body (1).

Already in the late 70s it was suspected that circadian rhythms might decisively influence stem cell functions and thus control both homeostasis and regeneration of peripheral tissues (2). Hematopoietic stem cells (HSCs) stand out as one of the most studied stem cell types. HSC functions are subjected to circadian fluctuations to ensure blood cell replenishment in mice and humans (3, 4). Although several studies have reported the expression of circadian clock genes (such as Bmal1, Clock, Cry1, Cry2, Per1, Per2, Rev-erb alpha, and Rev-erb beta) in mouse or human HSCs (5, 6), these genes do not exhibit clear light/darkness oscillatory patterns in HSCs (7). Indeed, a two-clock timing model was proposed to explain these differences in clock genes regulation (8). This model distinguishes between an endogenous clock, which is dominant in the neural system and is adjusted daily by photic signals, and an exogenous clock, which is more relevant for hemato-immune cells and is synchronized by environmental factors and/or changes in the organismal metabolism. According to this model, circadian rhythms in blood cells would be mostly regulated by the exogenous clock and rely on other oscillatory metabolic parameters and external cues (light-independent). However, as explained with more detail in the following sections, extensive research over the last few decades has demonstrated that photic cues and neural signals indeed regulate important circadian HSC functions in the bone marrow (BM) microenvironment.

The sympathetic branch of the autonomic nervous system connects the master clock in the central nervous system with peripheral organs to relay circadian information. For instance, adrenergic signals regulate clock gene expression in mouse liver (9), mouse heart (10), mouse and human osteoblasts (11, 12) and mouse brown adipose tissue (13). Similarly, β2-adrenergic receptor appears to regulate clock gene expression in BM stromal cells (14). Whilst adrenergic activity contributes to synchronize the peripheral oscillator, peripheral circadian rhythmicity can be maintained through other unknown mechanisms in the absence of adrenergic signaling (15).

Sympathetic nerves enter the BM associated with arteries and arterioles, and once inside the BM these nerves sprout throughout the BM space. BM sympathetic fibers mainly release noradrenaline in the BM, although both circulating noradrenaline and adrenaline can also reach the BM cavity via the vascular network (16). In sharp contrast, there is scarce neuroanatomical evidence of parasympathetic innervation in the BM. Although an indirect parasympathetic regulation of sympathetic postganglionary terminals cannot be excluded, the lack of parasympathetic fibers in the BM probably explains the scarce evidence for cholinergic regulation in the BM. It is important to note that not all autonomic cholinergic fibers are parasympathetic. In fact, some sympathetic fibers innervating the sweat glands or the periosteum undergo a neurotransmitter switch during postnatal development and start releasing acetylcholine (17, 18). Until recently, the role of these so-called sympathetic cholinergic nerve fibers in the BM has remained elusive. We have recently found that bone-associated cholinergic fibers play a crucial role in day/night oscillations of circulating HSCs and leukocytes. Indeed, sympathetic cholinergic fibers cooperate with the previously known sympathetic noradrenergic fibers and with central parasympathetic signals to orchestrate the circadian migration of HSCs and leukocytes between the BM and the bloodstream (19).

In this article, we discuss recent findings clarifying the roles of (nor)adrenergic and cholinergic signals to regulate different circadian HSC functions in BM niches. The future directions and perceived challenges for the future development of this multisystemic research are outlined below.

NEURAL REGULATION OF CIRCADIAN HSC TRAFFIC

The HSC is a unique type of adult stem cell since it preserves a remarkable migratory capacity at adulthood (20). HSCs circulate between the BM and the bloodstream in response to chemotactic signals, among which the C-X-C motif chemokine 12 (CXCL12) stands out as the most important chemokine. Although the biological function of HSC traffic is not yet fully understood, it is now accepted that HSCs circulate following circadian oscillations controlled by neural signals in different species, including humans (21, 22).

In the mouse system, sympathetic postganglionic terminals convey the circadian information from the suprachiasmatic nucleus to the BM, where noradrenaline is locally released. The adrenergic signals activate β3-adrenergic receptors in BM nestin+ mesenchymal stem cells (MSCs) and modulate CXCL12 production. This regulation leads to daily fluctuations in BM CXCL12 levels that inversely correlate with HSC numbers in the bloodstream. At daytime (the resting period in mice), light-induced noradrenergic signals downregulate CXCL12 expression via Sp1 transcription factor and promote HSC egress to the bloodstream. At night, BM CXCL12 levels are restored and HSCs preferentially return to the BM (also referred to as "HSC homing"). Remarkably, although circadian signals reach the BM through sympathetic terminals, their origin is set in the clock genes of the suprachiasmatic nucleus since normal circadian fluctuations are entrained by light and disappear in Bmal1$^{-/-}$ mice (21). Importantly, the expression of the CXCL12 receptor CXCR4 in HSPCs is also subjected to circadian oscillations synchronized with the ligand. In the human system, these circadian oscillations are also detected but inverted compared with mice, as expected from humans and mice respectively being diurnal and nocturnal species (22). Interestingly, mouse and human circulating leukocytes also exhibit opposite circadian oscillations when they coexist in humanized mice (23), suggesting that cell-autonomous mechanisms are involved in species-specific circadian regulation.

Different types of adrenergic receptors cooperate in the regulation of HSC traffic. Besides the described role for β3-adrenergic receptor, β2-adrenergic receptor appears to regulate clock gene expression in BM stromal cells and cooperates with β3-adrenergic receptor in HSPC mobilization induced

by granulocyte colony-stimulating factor (G-CSF), the agent most commonly used in the clinics (14). Interestingly, the same β-adrenergic receptor signaling was proposed to govern circadian leukocyte recruitment to mouse tissues (including the BM) during the nocturnal (active) phase in mice through stimulation of vascular endothelial cell adhesion (24). Therefore, these studies underscore the role of sympathetic noradrenergic signals as master regulators of circadian migration of leukocytes and HSCs/progenitors, which overall follow similar patterns. Daily neutrophil clearance by macrophages could be one event synchronizing leukocyte and HSPC trafficking. Macrophages are key cells to maintain HSCs in the BM (25, 26). Using mouse models, Casanova-Acebes et al. showed that aged neutrophils migrate at the end of the resting period to the BM, where they are engulfed by macrophages. This leads to the activation of LXR nuclear receptors on macrophages, which reduces the capacity of MSCs to retain HSPCs in the BM (27).

Despite these important findings, some critical aspects of circadian hematopoietic stem and progenitor cell (HSPC) traffic have long remained unanswered. For instance, A) how do BM stromal cells integrate similar noradrenergic signals at different circadian times and yet trigger distinct migratory processes (egress vs. homing)? In contrast to BM stroma-restricted expression of β3-adrenergic receptor, β2-adrenergic receptor is ubiquitously expressed (28, 29). As mentioned before, β2-adrenergic receptor cooperates with β3-adrenergic receptor in G-CSF-induced HSPC mobilization (14). Interestingly, adrenaline and noradrenaline can bind to β2-and β3-adrenergic receptors but they do so with very different affinities (30), suggesting that adrenaline and noradrenaline could have different effects on various cell types depending on the repertoire of adrenergic receptors expressed by the target cells. B) In that regard, do sympathetic neurotransmitters elicit similar responses if they signal in different BM cell populations or even through different β-adrenergic receptors in the same cell? C) Is noradrenaline the only neurotransmitter controlling circadian HSPC traffic?

An additional candidate neurotransmitter regulating HSPC traffic was hypothesized to be acetylcholine. Although modest, cholinergic innervation arising from the cholinergic forebrain and brain stem nuclei has been found in the suprachiasmatic nucleus. These cholinergic neurons have been proposed to play a role in the formation of time memory (memory of a specific time of the day associated with a certain event) (31). Recently, it was revealed that these cholinergic signals promote G-CSF-induced HSC mobilization from the BM through a glucocorticoid-signaling relay (32). Circulating glucocorticoids, such as corticosterone, follow circadian oscillations in blood and control HSPC proliferation through Notch signaling (33) and T cell distribution and activity through IL-7 receptor and CXCR4 (34). However, the precise role of circulating catecholamines in the circadian regulation of BM HSC niches was not well-understood.

In the context of physiological human hematopoietic cell trafficking between the BM and peripheral blood, one group of leukocytes (granulocytes, macrophages, natural killer cells, extrathymic T cells, gamma delta T cells and CD8+ cells) peak in the bloodstream at daytime, whilst another group (CD4+ T cells and B cells) exhibit an increase at night. Interestingly, those leukocytes with peak numbers in circulation at daytime express a higher density of adrenergic receptors, whereas those peaking at night carry a higher proportion of cholinergic receptors (35). Together, these data suggest a potential role for both adrenergic and cholinergic systems in the regulation of circadian hematopoietic cell trafficking.

Indeed, we recently showed that the autonomic cholinergic nervous system (including parasympathetic nerves but also a proportion of sympathetic neurons releasing acetylcholine) cooperates with (nor)adrenergic sympathetic signals to orchestrate day/night oscillations of circulating HSPCs and leukocytes (19). Our findings reveal a dual cholinergic signaling that plays different roles in mice at day and night (**Figure 1**).

At night, inhibitory central cholinergic signals (parasympathetic) dampen sympathetic noradrenergic tone to reduce nocturnal egress of HSPCs and leukocytes from the BM into bloodstream. Whether central parasympathetic circadian activity is regulated by core clock genes in the suprachiasmatic nucleus or elsewhere remains unknown. Collectively, the results suggest that this cholinergic regulation cooperates with adrenergic signaling to drive HSPC and leukocytes recruitment to the BM during the active phase (night for rodents, day for humans). We propose that plasma adrenaline, which follows circadian fluctuations peaking at night (36, 37), preferentially binds to β2-adrenergic receptors to promote vascular adhesion and BM homing at night. Supporting this possibility, BM mRNA expression of β2- and β3-adrenergic receptors oscillate peaking at night or day, respectively, and genetic or pharmacological blockade of each receptor demonstrated their circadian time-dependent roles in HSC or leukocyte BM egress/homing (19).

In mice, light cues trigger sympathetic noradrenergic activity, which signals through β3-adrenergic receptor leading to predominant BM egress of HSPCs and leukocytes in the morning (21). However, light exposure seems to equally activate a subset of bone-associated sympathetic cholinergic neurons (18), whose functions had remained elusive. Our data show that light-triggered sympathetic cholinergic signals inhibit BM vascular cell adhesion and homing, which enable predominant egress of HSPCs and leukocytes into bloodstream at this circadian time (19).

Selective predominance of different β-adrenergic signaling at daytime and night, together with differential affinities of catecholamines for β-adrenergic receptors, may explain how sympathetic noradrenergic signals trigger BM egress or homing at different time points. Furthermore, we propose that a robust circadian rhythmicity in β-adrenergic receptor expression in BM stromal cells allows for these fine-tuned oscillations. Whereas, β3-adrenergic receptor expression is higher after light exposure (when BM egress becomes overriding), β2-adrenergic receptor expression predominates at night (when BM homing is maximum) (19). An additional role for sympathetic cholinergic innervation in this circadian regulation appears to be the inhibition of β3-adrenergic receptor expression at night.

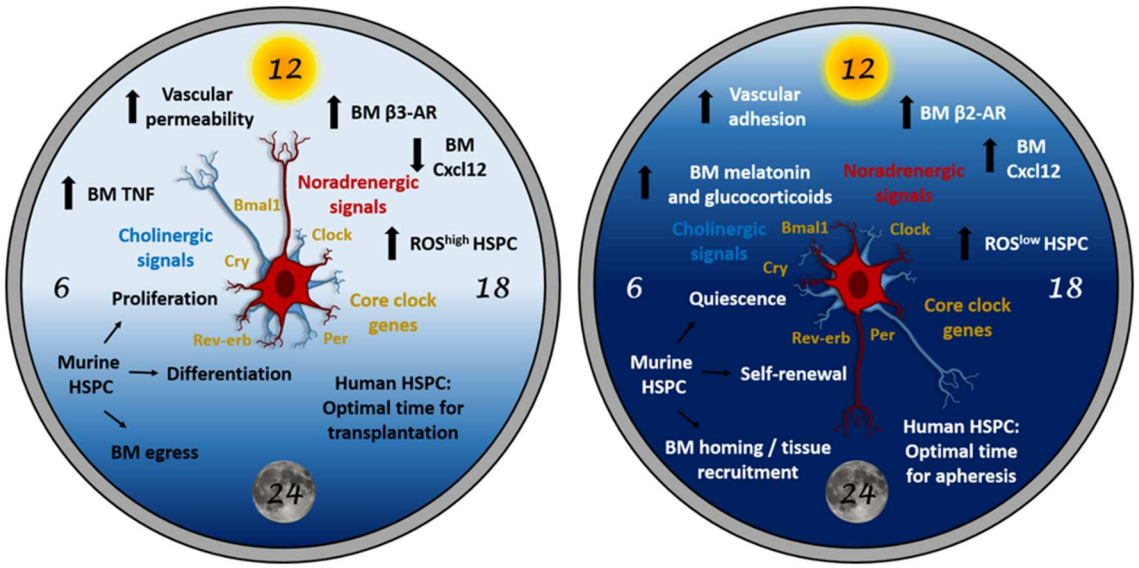

FIGURE 1 | The "circadian clock" for HSPCs. HSPC biology is subjected to light-entrained circadian regulation orchestrated by the central clock (suprachiasmatic nucleus in the brain) and relayed to the BM by neural signals (noradrenergic and cholinergic), hormones and cytokines. At daytime (left panel), these signals trigger mouse HSPC proliferation, differentiation and egress to the bloodstream. At night (right panel), similar signals trigger different responses in the BM microenvironment resulting in mouse HSPC quiescence, self-renewal and homing to BM or peripheral tissues. In humans (diurnal), circadian oscillations occur in antiphase with mice (nocturnal). Therefore, the HSPC harvest yield from peripheral blood would be higher during the evening, whereas the homing efficiency of the transplanted HSPCs would be higher in the morning in humans. Although the possible role of clock genes in HSPCs remains under-investigated and might not be essential under homeostasis, clock genes (which regulate cell cycle) in HSCs might be important during stress or in hematological malignancies.

NEURAL REGULATION OF CIRCADIAN HSC PROLIFERATION

Whilst it has been long appreciated that mouse and human HSPC proliferation (indirectly measured as DNA synthesis in the BM) follows a cyclic pattern, whether it is subjected to a bona-fide circadian rhythmicity has remained controversial for a long time (38–45). A potential role of the nervous system in this regulation was suggested after finding circadian fluctuations of catecholamines in human blood (46). Importantly, a pioneering study from Maestroni and colleagues had shown that noradrenergic stimulation promotes BM proliferation and protects against carboplatin-induced chemotherapy (47), which was later confirmed at the HSC level (48). Along these lines, noradrenaline concentration in the mouse BM correlates with the proportion of cycling hematopoietic cells (both peaking during the night or active phase for rodents), which altogether suggested that neural signals transduce the circadian information from the brain to the BM (16, 49). These observations also helped to understand the circadian-related differences in the capacity of murine HSPC to engraft in immunodeficient mice (50).

The Lapidot group confirmed these initial findings in humans using immature CD34+ cells (51). It is important to note that human and mouse hematopoietic cells appear to have a different clockwork, since inverted oscillations of circulating leukocytes have been described in immunodeficient mice carrying human and mouse leukocytes (52). Interspecies differences of stress-kinase regulation of reactive oxygen species (ROS), hypoxia-inducible factor 1α (HIF-1α) and clock gene–dependent regulation of the CXCL12 receptor CXCR4 appear to explain the opposite migratory patterns for human and mouse leukocytes (23). Noradrenaline and dopamine were found to increase human HSPC motility, proliferation, colony formation capacity and engraftment into immunodeficient mice (51). Interestingly, some neurotransmitters (such as noradrenaline) can exert these functions through directly activating β2-adrenergic receptors on hematopoietic cells (51). Therefore, the sympathetic nervous system regulates HSPC function through concerted actions on different receptors expressed by HSPCs (51) and their niche cells (19, 21).

The Lapidot laboratory has recently revealed the role of circadian BM noradrenergic signals in regulating the maintenance, proliferation and differentiation of mouse HSCs (53). Interestingly, two daily peaks of BM HSPC activity (one diurnal, one nocturnal) are preceded by transient increases of noradrenaline and tumor necrosis factor (TNF). Resembling circadian HSC traffic regulation through the BM niche (19, 21), the same signal appears to trigger different responses at each circadian time. For instance, TNF in the morning promotes HSPC proliferation, differentiation and migration, whilst TNF burst at night induces melatonin secretion to increase HSPC maintenance and self-renewal (53) (**Figure 1**). Overall, sympathetic noradrenergic signals cooperate with TNF levels to orchestrate blood cell replenishment during the day, whilst they serve to maintain the BM HSPC pool at night. The noradrenaline-TNF pathway promotes opposite

HSC functions through a combination of mechanistic players including differential regulation of reactive oxygen species, vascular permeability, antigen expression and macrophage function. Based on the important contribution of the cholinergic neural system to day/night oscillations in HSPC and leukocyte migration (19), the possible regulation of HSPC proliferation and differentiation by cholinergic signals will be studied in the future.

A BIOLOGICAL MEANING FOR CIRCADIAN HSC REGULATION

The idiosyncrasy of the central master clock in mammals suggests that the biological benefit of these circadian oscillations might reside in its capacity to integrate and orchestrate different physiological functions that require precise coordination in order to adapt to organismal demands. The bone organ (including bone and BM) might actually represent a paradigmatic example of integrated physiology (54), since research over the past few decades has shown that bone and BM are coordinately regulated. For instance, whilst β3-adrenergic receptor was originally described to control circadian HSC traffic between the BM and the bloodstream, β2-adrenergic receptor has a prominent role in the circadian proliferation of bone-forming osteoblasts (11). Sequential proliferation and differentiation of MSC and osteolineage cells occurs at distinct circadian times. In rodents, preosteoblasts, the immediate proliferating precursors of osteoblasts, synthesize DNA primarily during the light cycle and divide during the subsequent dark cycle, whereas more immature osteoprogenitors display an inverted cycle (55). Perivascular nestin+ MSC are in contact with sympathetic fibers and are targeted by sympathetic efferent activity, whereas preosteoblasts are distributed closer to the bone surface (56). In addition, β3-adrenoceptor activation in nestin+ MSC inhibits their osteoblastic differentiation, while it does not affect their proliferation (56). In BM stromal cells, like osteoblasts, β2-adrenoceptor stimulation induces clock gene expression, whereas activation of β3-adrenoceptor downregulates *Cxcl12* (14). These data, together with the increased proliferation of nestin+ MSC after chemical sympathectomy (56), suggests that β2-adrenoceptor activation is responsible for circadian induction of nestin+ MSC proliferation, like previously reported for preosteoblasts (11). Therefore, it is possible that the differential localization, innervation and expression of β-adrenoceptors allow for circadian coordinated regulation of proliferation and differentiation of preosteoblasts and MSC, the last directly affecting HSC maintenance in the BM.

More broadly, many cytokines regulating bone accrual and bone formation work in a circadian fashion (with higher bone formation rate during the day in rodents) (57, 58). Therefore, since both systems are closely associated and even share multiple cell types and molecular pathways, it is reasonable to hypothesize that their circadian regulation is a control mechanism to ensure the coordinated and homeostatic function of the bone organ as a whole.

Alternatively, it has been proposed that daily HSC egress to circulation during the resting phase (light in rodents and night in humans) might contribute to regenerate BM stem cell niches (22, 59). Circulating HSCs would migrate to extramedullary tissues, where they could differentiate into mature hematopoietic cell types (60). It is important to note that the number of circulating HSCs at any time is very low, hampering the demonstration of their biological function. Furthermore, it is difficult to separate the impact of circadian rhythms *per se* from the physiological processes undergoing oscillations. For instance, sleep deprivation in mice reduces by 50% the capacity of HSCs to engraft into recipient mice undergoing normal day/night sleep cycles (61), but it is difficult to discern the effects of altered biological oscillations from the lack of regeneration due to sleep deprivation.

Another possible function of circadian HSPC traffic would be to maintain a pool of circulating progenitor cells to anticipate potential requirements derived from stress situations. In this sense, inflammatory monocytes have been found to follow circadian oscillations. However, this was shown to be mediated by intrinsic Bmal1 expression in myeloid cells, rather than light or environmental cues (62). In the aging context, some adult stem cells such as epidermal, muscle or liver stem cells do not lose their core circadian machinery, but their transcriptome switches from homeostasis genes to tissue-specific stress genes (e.g., DNA damage or autophagy) to adapt to age-related traits (63, 64). Supporting this idea, we found an adrenergic signaling switch in the aged BM niches from predominant β3- to β2-adrenergic receptor signaling, which facilitates myeloid cell expansion during aging (65).

As indicated above, clock genes in HSCs do not seem to play a crucial role in the homeostatic regulation of healthy HSCs but, as gatekeeper regulators of cell cycle, they can acquire a prominent role during stress (e.g., cancer, inflammation, etc.) (66). In contrast to healthy HSCs, the expansion of leukemic stem cells seems to be highly dependent on core clock genes. Bmal1 and Clock transcription factors were reportedly essential for leukemic stem cell self-renewal in acute myeloid leukemia (66). Therefore, anti-carcinogenic chronotherapies might take advantage of this susceptibility factor to target cancer stem cells without affecting normal stem cells. Furthermore, BM neuropathy is critical for the development of myeloproliferative neoplasms and acute myelogenous leukemia (67, 68), whilst altered noradrenergic signaling has been reported in aging (65, 69). Since neural signals are subjected to circadian fluctuations in the healthy/homeostatic BM, the disruption of neural circadian regulation might also contribute to disease/aging development, and thus represent a niche-related target for chronotherapies. In general, chronotherapy approaches in cancer have proven much more difficult than expected probably due to the interplay of different clocks and multiple entrainment signals in humans as a cause of notorious heterogeneity (52). It is important to note that the clock genes comprise only one of several cellular clocks. Other studies have found clock gene–independent mechanisms that maintain circadian cycles. For instance, ROS-regulating enzymes, such as peroxiredoxins, are ancient clocks that have been conserved throughout evolution (70). Therefore, future efforts in chronotherapy should take into account the

interplay between the genetic clock and metabolic clocks, for instance.

POTENTIAL CLINICAL IMPLICATIONS OF CIRCADIAN HSC RHYTHMS

Since both circadian HSC proliferation and migration can impact HSC numbers in circulation, on a practical note it is recommended that clinicians consider harvesting blood (apheresis) later during the day to increase the stem cell yield in cases where HSC numbers might be limiting for the success of HSC transplantation procedures. Based on experimental evidence, a 2-3-fold higher HSPC number can be harvested by apheresis in the evening, compared with the morning apheresis, even after G-CSF-enforced HSC mobilization (22, 71). In contrast, it is expected that human HSC homing and subsequent engraftment in the BM would be more efficient early in the morning, when the BM transplantation procedure would be recommended for "poor engrafters" (24).

CONCLUSIONS AND PERSPECTIVES

In this article, we have summarized our current knowledge of the circadian regulation of HSPCs with a particular focus on the important yet intriguing role of sympathetic noradrenergic signals promoting HSPC proliferation, differentiation and egress during the resting phase (daytime for rodents), and HSPC maintenance, self-renewal and BM homing during the active phase (night for rodents) (**Figure 1**). The concerted regulation of HSPC function by noradrenergic signals requires fine-tuned control of adrenergic receptor expression, cytokines (e.g., TNF), hormones (like adrenaline or melatonin), ROS, vascular adhesion and permeability and, more broadly, BM HSC niche cells, such as MSCs, endothelial cells and macrophages. Moreover, the noradrenergic sympathetic system does act alone but cooperates daily with peripheral sympathetic cholinergic fibers and with central parasympathetic tone to orchestrate this complex HSPC regulation (19). A fascinating interplay among different organs takes place between brain, bone and BM to ensure that each organ's response meets the organismal demands.

The pathophysiological implications of the internal HSC clock remain largely unknown. In other systems, like the epidermis, the molecular clock machinery underlies the heterogeneity in the epidermal stem cell pool and could directly impact cell fate decisions, such as proliferation vs. dormancy. Particularly, differential clock gene expression leads to distinct responses of epidermal stem cells to transforming growth factor (TGF)-β and Wnt, which are signals that normally sustain their quiescence or proliferation, respectively (72). Furthermore, single-cell technology has revealed clock gene-dependent metabolic oscillations coordinated with DNA synthesis in these proliferating stem cells (73). It is possible that some of these regulatory mechanisms of epidermal stem cells are shared with HSCs, but this remains to be investigated. By the same token, the principles of neural regulation of HSC niches might be extrapolated in the future to other tissues, including the epidermis.

Whilst the influence of the neural system on HSPC differentiation has already been noted (53), the molecular drivers of cell commitment deserve further investigation. Likewise, cross-fertilization of ideas with other stem cell systems might pave the way for candidate molecular pathways involved. For instance, in both mouse and human embryonic stem cells, the *in vitro* pluripotent potential inversely correlates with the establishment of a circadian clock machinery, suggesting a function of the clock genes during embryonic stem cell differentiation (74, 75). Comparatively, the differentiating signals possibly driving the emergence of the clock core genes in adult stem cells are poorly understood (76). The potential role of neural signals either triggering and/or entraining clock-dependent differentiation of HSCs remains to be investigated.

Finally, cumulative evidence points toward the interplay among circadian clock system disruption, stress situations and multiple systemic diseases (77). In the hematopoietic field, unraveling the contribution of neural signals to HSC circadian regulation under acute or chronic stress might help to develop novel chronobiological therapies for hematological disorders based on neuroactive agents. As Hippocrates quoted: "Healing is a matter of time, but it is sometimes also a matter of opportunity."

AUTHOR CONTRIBUTIONS

AG-G prepared the figure. AG-G and SM-F authors wrote the manuscript.

FUNDING

Original work discussed in this article was supported by core support grants from the Wellcome Trust (203151/Z/16/Z) and the Medical Research Council (MRC) to the Cambridge Stem Cell Institute, the Spanish Ministry of Economy and Competitiveness (SAF- 2011-30308), the Pro-CNIC Foundation, Severo Ochoa Center of Excellence award SEV-2015-0505 to CNIC, Ramon y Cajal Program grant RYC-2009-04703, Marie Curie Career Integration grant FP7-PEOPLE-2011-RG-294096, ConSEPOC-Comunidad de Madrid S2010/BMD-2542, Red TerCel (Instituto de Salud Carlos III (ISCIII)-Spanish Cell Therapy Network), National Health Institute Blood and Transplant (United Kingdom), Horizon2020 grant ERC-2014-CoG-648765, and a Cancer Research UK Programme Foundation Award (C61367/A26670) to SM-F, who was also supported in part by an International Early Career Scientist grant from the Howard Hughes Medical Institute. AG-G received fellowships from the Ramon Areces and LaCaixa Foundations, and is currently supported by the Swiss National Science Foundation (SNSF 31003A-179259). The authors apologize for the omission of relevant literature due to space limitations.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 12 June 2020
doi: 10.3389/fimmu.2020.01237

Circadian Regulation of the Biology of Allergic Disease: Clock Disruption Can Promote Allergy

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Allergic diseases such as allergic rhinitis, asthma, atopic dermatitis, and food allergy are characterized by epithelial barrier dysfunction and deregulated immune responses. Components of the circadian clock interact with critical elements of epithelial barrier function and immune responses, and regulate the biological processes on a 24-h cycle at steady state. This may represent an anticipatory defense response to day–night fluctuation of attack by noxious stimuli such as pathogens in the environment. This review will summarize clock control of epithelial barrier function and immune responses associated with allergic disease and offer novel insights and opportunities into how clock dysfunction impacts allergic disease. Importantly, perturbation of normal clock activity by genetic and environmental disturbances, such as chronic light cycle perturbations or irregular eating habits, deregulates epithelial barrier function and immune responses. This implies that the circadian clock is strongly linked to the fundamental biology of allergic disease, and that clock disruption can precipitate allergic disease by altering the epithelial barrier and immune functions. Given that contemporary lifestyles often involve chronic circadian disruptions such as shift work, we propose that lifestyle or therapeutic interventions that align the endogenous circadian clock with environmental cycles should be a part of the efforts to prevent or treat allergic disease in modern society.

Keywords: circadian clock, allergic disease, epithelial barrier function, immune response, clock disruption

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
David Ray, University of Oxford, United Kingdom
Anne Dudeck, Universitätsklinikum Magdeburg, Germany

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *25 March 2020*
Accepted: *18 May 2020*
Published: *12 June 2020*

Citation:
Nakao A (2020) Circadian Regulation of the Biology of Allergic Disease: Clock Disruption Can Promote Allergy. Front. Immunol. 11:1237. doi: 10.3389/fimmu.2020.01237

INTRODUCTION

Allergic diseases such as allergic rhinitis, asthma, atopic dermatitis, and food allergy are serious public and medical concerns due to their high prevalence and the harm they cause, in terms of both patient quality of life and socioeconomics (1). However, we do not fully understand why allergic disease is so prevalent in modern society. Hence, we need to understand previously unrecognized aspects of the biology of allergic disease.

Allergic disease is characterized by epithelial barrier dysfunction and deregulated immune response (allergic immune response) (2–4). For instance, in atopic dermatitis (AD), several components of skin barrier function, such as filaggrin, tight junction, and the microbiome are compromised in terms of quantity and/or quality, leading to increased cutaneous permeability (5). Consequently, the skin permits allergen penetration and releases epithelial cytokines (e.g., IL-33), which trigger an allergic immune response. Decreased regulatory T-cell activity in AD may also promote allergic immune responses (6).

The circadian clock is the endogenous timing-keeping mechanisms, by which living organisms fit their physiology and behavior to daily alterations in the rhythmic environment created by the earth's rotation (7). Recent studies highlight that the circadian clock underpins epithelial barrier function and immune responses under physiological conditions (8–12). The components of the circadian clock interact with critical elements or pathways of epithelial barrier function and immune responses, thereby regulating these biological processes on a 24-h cycle. This may represent an anticipatory defense response to day–night fluctuation of attack by pathogens and non-pathogenic insults in the outer environment (11, 13). In this review, I will summarize clock control of epithelial barrier function and immune responses that are associated with allergic disease and provide insight into how clock dysfunction affects allergic disease. Allergic disease is also well-characterized by marked day-night changes in the clinical symptoms, laboratory parameters, and response to treatment. Readers who are interested in this subject are encouraged to refer to the reviews on how the circadian clock underpins a time of day–dependent variation in allergic reactions (14–16). The reviews highlight that oscillatory allergic reactions are generated by rhythmic expression of key molecules in the pathophysiology controlled by the circadian clock.

MOLECULAR CLOCKS IN MAMMALIAN CELLS

In mammals, the circadian clock consists of several clock genes that are expressed in virtually all cell types, including cells in the skin, gut, airways, and immune system (11, 12, 17–19). At its core, the molecular clock consists of interlocking transcriptional–translational feedback loops (TTFLs) centered on the transcription factors BMAL1 and CLOCK (20, 21). BMAL1 heterodimerizes with CLOCK, and the heterodimer binds to E-box motifs (CANNTG) throughout the genome, driving the expression of thousands of genes, including *Period (Per1-3)* and *Cryptochrome (Cry1,2)*. The PER and CRY proteins form oligomers and move to the nucleus, where they inhibit BMAL1/CLOCK activity. This core loop, which takes ∼24 h to complete, involves several post-transcriptional mechanisms such as enzymatic degradation of PER and CRY, and acts as a molecular oscillator to represent time of day within each cell.

Other than this core loop, a stabilizing loop within the clockwork regulating the timing and amplitude of BMAL1 is provided by the nuclear receptors RORα and REV-ERBα (Nr1d1) (20, 21). The BMAL1/CLOCK heterodimer activates transcription of RORα and REV-ERBα, which respectively activates and represses BMAL1 transcription.

Accordingly, the network of circadian proteins mediates periodic expression of thousands of genes (clock-controlled genes: CCGs) and regulates the timing of cellular activities on a 24-h cycle, ultimately dictating rhythmic physiology in various organs. The impact of the clockwork machinery is enormous: many CCGs, such as nuclear hormone receptors (NRs) like glucocorticoid receptor, are key regulators of major physiological processes (e.g., metabolism, immunity, development, reproduction) (22). Overall, 43% of all protein coding genes in mice shows circadian rhythms in transcription somewhere in the body, in an organ-specific manner (23).

COORDINATION OF THE MULTI-OSCILLATORS

The human body contains ∼40 trillion cells, each with a ∼24-h clock. Thus, our body consists of a multi-oscillator network system. How are these astronomical numbers of clocks synchronized with other?

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus (the central clock) serves as the master pacemaker in the body (20, 21, 24). The SCN receives afferent innervation about environmental light levels from the retina via the retinohypothalamic tract, which synchronizes the SCN clock via the cAMP response element binding (CREB) protein. In turn, the SCN transmits neuronal (e.g., sympathetic nerve activity) and hormonal (e.g., cortisol) signaling to synchronize peripheral clocks.

However, peripheral clocks can also be reset by non-SCN-derived hormones (e.g., insulin) and metabolic (e.g., NAD^+ [nicotinamide adenine dinucleotide]$^+$) signals connected with non-photic environmental cues such as food timing (25). For instance, livers of mice fed exclusively during the night or *ad libitum* (active phase) show a similar phase angle of cyclic liver gene expression, whereas feeding during the day almost entirely inverts the phase of liver oscillatory gene expression (26).

HOW DOES CIRCADIAN DISRUPTION OCCUR?

As stated, circadian clocks become synchronized to a 24-h periodic environmental cue, which is called the zeitgeber ("time-giver" in German). Light and meal timing are strong zeitgebers. Accordingly, circadian rhythms in behavior, physiology, and metabolism become robust when the rhythmicity of internal clocks is coupled to that of external zeitgebers (20, 21, 24, 25). In other words, rhythms in the circadian system dampen when internal clock timing becomes mismatched with environmental zeitgebers.

Misalignment between the endogenous circadian clock and environmental cycles (or zeitgebers) compromises human mental and physical health (27). For instance, chronic circadian misalignment via night shift work, jet lag, or exposure to artificial light at night can precipitate or exacerbate mood disorders in susceptible individuals (28). Notably, night shift workers, who are exposed to aberrant light/dark conditions, irregular eating habits, and sleep disruption, are at a higher risk of cancer, cardiovascular and metabolic diseases, as well as sleep/psychiatric disorders (29). Thus, dramatic changes in modern lifestyles, including night shift work, nocturnal feeding, and shortness or irregularity of sleep impair our health via chronic circadian misalignment.

CIRCADIAN REGULATION OF THE BIOLOGY OF ALLERGIC DISEASE

Clock Control of Epithelial Barrier Function Related to Allergic Disease

The epithelium in the skin, intestine, and airways acts as a physical, chemical, and biological barrier against pathogens, chemical agents, and allergens. Epithelial barrier dysfunction is critical for the initiation of allergic disease in many organs (2, 3, 5). In brief, disruption of epithelial barrier function increases epithelial permeability that enables entry of allergens into the body and activates the allergic immune response. Genetic (e.g., filaggrin deficiency) and non-genetic factors (e.g., protease activity of allergens, chemical agents, and injury/itch) contribute to the barrier disruption associated with allergic disease (30).

Below, I briefly summarize some examples of clock control of epithelial barrier function related to allergic disease. I will not discuss clock control of commensal bacteria, an important barrier against pathogenic and non-pathogenic insults in the epithelium of the skin, intestine, and airways, because this subject has been adequately reviewed elsewhere (31–33).

Skin

Our largest organ, the skin, is continuously exposed to numerous environmental irritants, including allergens. The skin barrier consists of many physical, chemical, and biological components, including filaggrin, lipid (e.g., ceramide), skin pH, tight junctions (TJs), anti-microbial peptides, commensal bacteria, and water content, most of which exhibit circadian rhythms (3, 34). In atopic dermatitis (AD), skin barrier function is impaired by several mechanisms, including filaggrin deficiency, injury (itch), and type 2 cytokines (e.g., IL-4 and IL-13) altering TJ protein expression, thereby precipitating allergic sensitization and inflammation (34, 35).

A clear example of circadian control of skin barrier function is aquaporin-3 (AQP3), which regulates water content by facilitating water and glycerol entry into keratinocytes. AQP3 expression in the skin is temporally controlled by CLOCK in mice and humans (36). Accordingly, stratum corneum hydration exhibits a significant 24-h rhythm in wild-type mice that is absent in *Clock*-mutated mice. Interestingly, *Clock*-mutated mice exhibit persistently reduced stratum corneum hydration (36). These findings illustrate that clock disruption can deregulate the components of skin barrier function.

Intestine

Intestinal epithelial cells function as the first line of defense against pathogenic and non-pathogenic microorganisms in the gut lumen (37). These epithelial barriers include TJs, mucus, and anti-microbial peptides. Several reports in humans and mice suggest that food allergy is associated with intestinal barrier dysfunction, which increases intestinal permeability to food compounds (38).

Several studies report that intestinal barrier function is under the circadian control. The epithelial TJ is a multiprotein complex that forms a selectively permeable seal between adjacent epithelial cells and limits paracellular passage of macromolecules, including allergens (39). CLOCK regulates expression of the TJ molecules Occludin and Claudin-1 in the mouse colon and controls circadian periodicity of intestinal permeability (40). In addition, in a mouse model of ovalbumin (OVA)-induced food allergy, OVA-induced allergic diarrhea exhibits daily variations associated with circadian periodicity in intestinal permeability (41), which implicates that the timing of food antigen intake can affect the severity of food allergy symptoms via clock control of intestinal permeability.

Importantly, *Clock*-mutated mice express lower levels of Occludin and Claudin-1 in the colon and are more sensitive than wild-type mice to the colonic injury induced by dextran sodium sulfate (DSS) (40). In addition, in a mouse model of chronic alcohol feeding, circadian disruption through genetics (*Clock* mutation) or environmental disruption (weekly 12-h phase-shifting) results in gut leakiness and exacerbates alcohol-induced gut leakiness and liver pathology (42). These findings illustrate that clock disruption can impair intestinal barrier integrity.

Airways

The airways are continuously exposed to physical, chemical, and biological insults in the air, including pathogens, pollutants, and allergens, and protect the host against them. Like the skin and intestine, the airway epithelial barrier consists of many physical, chemical, and biological components, including TJs, mucus-producing goblet cells, ciliated cells, and epithelial-derived cytokines/chemokines. Defects in the epithelial barrier are associated with asthma: for instance, bronchial biopsies from patients with asthma exhibit patchy disruption of TJs (43). Furthermore, these patients are more susceptible than healthy people to cigarette smoke or viral infection, which may precipitate asthma (44).

Pulmonary epithelial cells release cytokines and chemokines upon exposure to bacteria and viruses as a part of an innate defense response. The LPS-induced lung inflammatory response exhibits circadian periodicity in mice, relying on Bmal1 in bronchial epithelial cells (45). Since the epithelial response to LPS in the lung determines the development and severity of asthma (46, 47), Bmal1 regulation of the epithelial response to LPS may potentially explain the circadian nature of this disease.

Importantly, *Bmal1* deletion in bronchial epithelial cells enhances the LPS response in the lung without time-of-day oscillations (45). In addition, a loss-of-function mutation of REV-ERBα, a downstream target of Bmal1, in bronchial epithelial cells augments the LPS-induced inflammation in the lung, suggesting REV-ERBα as a key molecule that couples the core clock gene Bmal1 to innate immunity in the lung (48). Furthermore, responses to influenza infection are disrupted in mice with selective *Bmal1* deletion in bronchial epithelial cells (49). Thus, clock disruption can deregulate airway defense (barrier) mechanisms.

Clock Control of Innate Immune Responses Related to Allergic Disease

Mast Cells/Basophil Response

Mast cells and basophils are innate immune cells that share many features, although they constitute a distinct lineage

having several different roles in immune response and tissue homeostasis. In IgE-mediated allergic diseases such as allergic rhinitis, asthma, and food allergy, mast cells and basophils are the main effector cells and are activated by an IgE-dependent mechanism. IgE produced in response to allergen binds to the high-affinity IgE receptor (FcεRI) on the cell surface of mast cells and basophils, which triggers degranulation and production of cytokines/chemokines and lipid mediators, thereby shaping allergic inflammation (50).

The circadian nature of IgE-mediated allergic diseases is well-documented: in allergic rhinitis and asthma, symptoms, nasal or bronchial reactivity, and inflammatory activity become more pronounced in the early morning and at midnight (51). Skin hypersensitivity to allergens also varies with the time of day (52). These findings suggest that IgE-mediated allergic disease is under circadian control.

Consistent with the clinical observations, CLOCK controls the expression of FcεRI in mouse mast cells and releases mediators in a circadian manner upon IgE stimulation (53). Human basophils isolated from patients with asthma or allergic rhinitis exhibit a time-of-day–dependent variation in IgE-mediated responses (54, 55), although these findings may be controversial (56). *In vivo*, the extent of the passive cutaneous anaphylactic (PCA) reaction, a classical rodent model of IgE/mast cell-mediated allergic reaction, exhibits circadian variations (53, 57, 58).

Interestingly, the extent of the PCA reaction in *Per2*-mutated mice, mast cell–selective *Clock*-mutated mice, or mice fed only in the resting phase persistently exhibits a peak level equivalent to that of wild-type mice, but without circadian periodicity (53, 57–59). Thus, circadian disruption due to genetic alteration or irregular eating habits increases the susceptibility of mast cells to IgE throughout the day.

IL-33 activates mast cells and basophils via its receptor ST2. The crucial roles of the IL-33/ST2 axis in both IgE- and non-IgE–mediated allergic disease have been appreciated (60). CLOCK temporally gates the mast cell and basophil response to IL-33 via regulation of ST2 expression, which may also underlie circadian nature of allergic disease (61). Importantly, *Clock* mutation persistently enhances their response to IL-33 (61). Thus, clock disruption may enhance IgE- or IL-33-mediated mast cell/basophil responses.

Eosinophil Response

Eosinophils are main effector cells for the control of parasitic infections, but increasing evidence suggests that they play regulatory roles in tissue homeostasis/repairs and adaptive immune response. In allergic disease, eosinophils mediate their effector functions through several mechanisms: degranulation, extracellular traps, and cytolysis (62). Eosinophils require IL-5 survival signals, and anti-eosinophil monoclonal antibodies targeting IL-5 or IL5 receptor (IL5R) have been approved for clinical use against eosinophilic asthma (63).

Human eosinophils exhibit circadian oscillations in basal ECP (eosinophil cationic protein) expression and IL-8 (CXCL8) and CCL2 (The chemokine [C-C motif] ligand 2) release upon fMLP (N-formyl-methionyl-leucyl-phenylalanine) stimulation (64, 65). Furthermore, the number of blood and sputum eosinophils or serum IL-5 exhibits a time-of-day–dependent variation in asthma and control subjects or in mice (66–68). Interestingly, circadian variation of blood eosinophils has been linked to neuroendocrine and metabolic cycling (68). These findings are consistent with circadian control of eosinophil homeostasis, activation, and migration under steady states and in allergic disease, although few studies have addressed the direct roles of the clock in eosinophil function.

Macrophage Response

Macrophages are versatile innate immune cells that have phenotypic diversity and function in many different aspects of physiology and disease. Generally, the roles of macrophages in allergic disease remain obscure, but, in allergic asthma, macrophages may promote inflammatory responses associated with lung injury, fibrosis, and goblet cells hyperplasia (69).

Circadian clock components regulate various functions in macrophages, including cytokine secretion upon LPS challenge (70–72). About 8% of genes expressed in peritoneal macrophages are rhythmically transcribed, including essential elements in LPS/TLR4 signaling (71).

Interestingly, mice with *Bmal1*-deficient myeloid cells have markedly elevated eosinophil accumulation and serum BALF IL-5 expression in a model of allergic asthma (73). *Bmal1*-deficient macrophages produce more asthma-associated CCL2 and CXCL10 upon LPS stimulation than wild-type macrophages. Further, targeting REV-ERBα in myeloid cells exhibits persistently high pulmonary neutrophilic inflammation upon LPS challenge without time-of-day-dependent variations (48). Thus, clock disruption may enhance asthma by altering responses to LPS in macrophages, as well as in bronchial epithelial cells.

Innate Lymphoid Cell Response

Innate lymphoid cells (ILCs) are the most recently discovered family of innate immune cells that are ubiquitously distributed and are enriched in mucosal surface. ILCs consist of three different groups: group1, group 2, and group 3 ILCs (ILC1s, ILC2s, ILC3s), based on transcription factors required for the development, cytokine expression, and distinct effector functions.

ILC2s are key regulators of allergic immune responses (74). The epithelium-derived cytokines IL-25, IL-33, and TSLP activate ILC2s. The activated ILC2s release IL-5 and IL-13, which initiate and amplify allergic inflammation by activating eosinophils and epithelial cells. ILC2s in blood are more abundant in patients with asthma than in healthy subjects (75). The roles of clock in ILC2s remain unclear, but it is highly likely that ILC2s development and function are under circadian control, since RORα, a component of circadian clock, is involved in ILC2 differentiation (76) and IL-5 production from intestinal ILC2s exhibits circadian rhythms associated with blood eosinophil counts (68).

In contrast to ILC2s, several studies suggest that ILC3s, which provide mucosal defense through IL-22 and IL-17, are under strong control of the circadian clock. Bmal1 deficiency in ILC3s or disruption of light–dark cycles and feeding rhythms can deregulate gut ILC3 homeostasis, impairs epithelial reactivity,

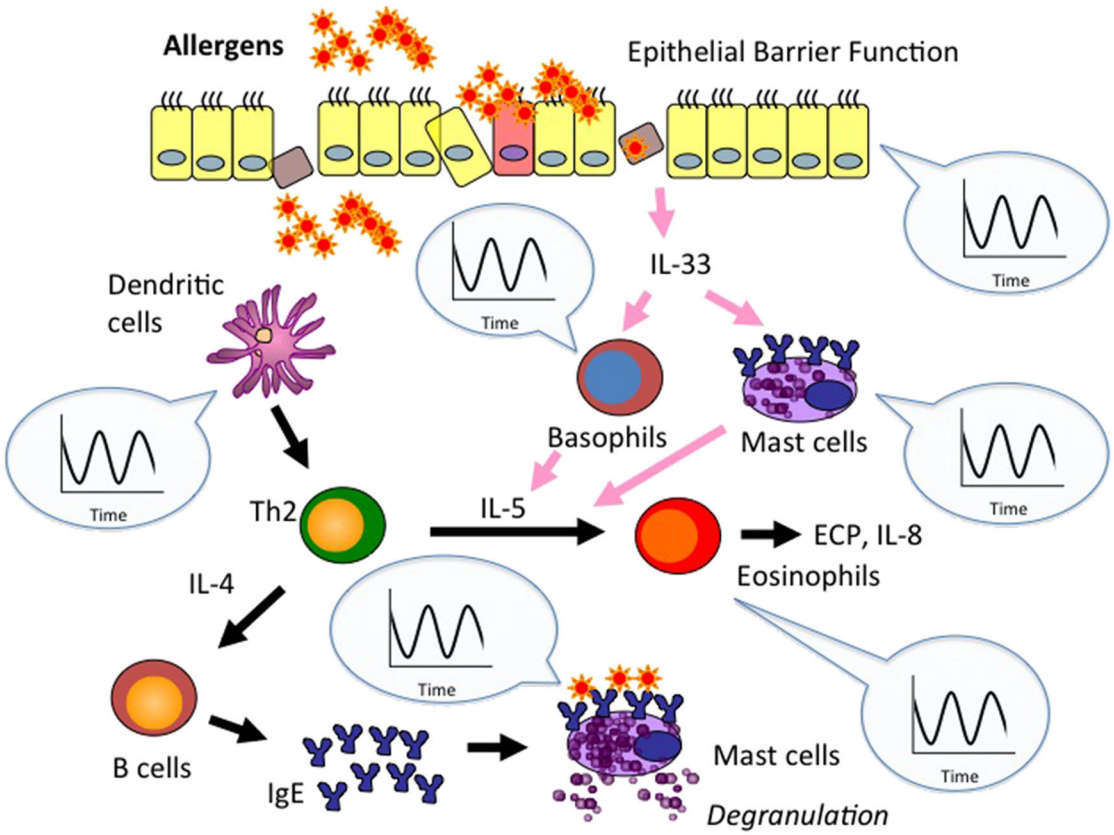

FIGURE 1 | Circadian clock activity is embedded in epithelial barrier function and immune response associated with allergic disease. Epithelial barrier disruption enables entry of allergens into the body and leads to production of innate cytokines such as IL-33. The cytokines skew dendritic cell (DC) phenotypes, activate innate immune cells (e.g., mast cells, basophils), and promote Th2 development. Th2 cells secrete IL-4 and IL-5, which promote IgE production from B cells and eosinophil accumulation, respectively, and shape allergic inflammation. As stated in the text, circadian clock activity is embedded in the control of epithelial barrier function and immune cell responses associated with allergic disease. Circadian control of allergy-related epithelial barrier functions and innate immune cell responses (e.g., mast cells, basophils, eosinopihls, dendritic cells) has been extensively studied so far. Thus, we emphasize clock control of these elements in this figure. Please note that macrophage-, innate lymphoid cell-, T cell-, and B cell-responses are also controlled by the circadian clock. Accordingly, clock disruption may predispose allergic disease by deregulating epithelial barrier function and immune cell responses.

and deregulates the microbiome (77, 78). Because ILC3s have been implicated in obesity-induced asthma (79), clock disruption through metabolic disturbances in obese patients might enhance ILC3 responses and predispose patients to asthma.

Dendritic Cell Response

Dendritic cells (DCs) are the most potent professional antigen-presenting cells (APCs) *in vivo*, which can induce the activation and differentiation of naive T cells or induce immune tolerance. The ability of DCs to trigger immunity or tolerance by their co-stimulatory/inhibitory molecules is likely involved in the development of allergic disease. For instance, DC-expressing semaphorin 4, one member of the large family of secreted and membrane-bound glycoproteins that were initially implicated in axon guidance and neural development, drives Th2 response and are highly expressed in human asthmatic lung tissue (80).

The roles of clock in DCs remain largely unclear. One study shows that deletion of *Bmal1* in dendritic cells enhances the gut helminth *Trichuris muris* egg-specific protective Th2 response while suppressing the Th1 response in mice, suggesting that circadian machinery in DCs may contribute to Th1/Th2 balance (81). Thus, disruption of the DC-clock may affect Th1/Th2 balance, thereby having impact on allergic disease.

Clock Control of Adaptive Immune Responses Related to Allergic Diseases

Lymphocytes (T cells and B cells) express clonally distributed receptors specific for diverse antigens (allergens) and are the key mediators of immune response. Among T cells, $CD4^+$ T cells are called helper T cells (Th cells) because they help B cells or phagocytes to produce antibodies or destroy ingested microbes, respectively. Further, $CD4^+$ T cells exhibit functionally distinct subsets called Th1, Th2, and Th17 that produce different cytokines and eliminate different types of pathogens.

In allergic disease, epithelial barrier disruption leads to production of innate cytokines such as IL-33 that skew DC phenotypes and activate innate immune cells (e.g., ILC2s, mast cells, and basophils), thereby eventually promoting the

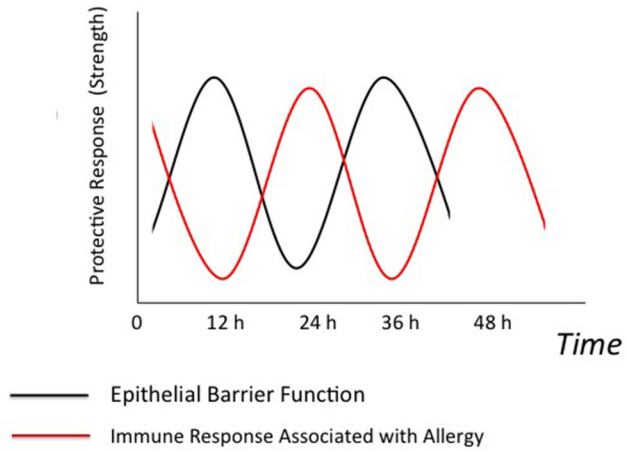

FIGURE 2 | Possible temporal relationships between epithelial barrier function and allergic immune response. Epithelial barrier function and immune response associated with allergic disease (allergic immune response) are under control of the circadian clock. We speculate that epithelial barrier function and allergic immune response might temporally complement to each other. Briefly, clock system may maximize allergic immune response at the time-of-day when epithelial barrier function is weakest. This may be because the risk of attack by helminth and biting arthropods is highest at that time.

development of Th2 cell that secrete IL-4, IL-5, and IL-13. These cytokines promote B-cell isotype switching and IgE production, eosinophil accumulation, and mucus production from epithelial cells and shapes allergic inflammation (2).

Clock genes are rhythmically expressed in both T and B cells (82, 83) and temporally control lymphocyte trafficking and development, and immune responses against diverse pathogens (10–13). However, there have been limited studies addressing the roles of clock in T and B cell responses associated with allergic disease.

T Cell-Response

A mouse model of contact hypersensitivity in the skin depends on Th2 cells. In this model, *Clock*-mutant mice exhibit severe inflammation and increases in IL-4/IL-13 expression, mast cell number, and serum IgE levels relative to wild-type mice (84). As stated earlier, deletion of *Bmal1* in dendritic cells enhances the gut helminth *Trichuris muris* egg-specific protective Th2 response while suppressing the Th1 response. These findings suggest that clock proteins inhibit Th2 development and function, and that clock disruption may promote the Th2 response.

Th17 cells protect against bacterial and fungal infections at mucosal surfaces. In contrast to Th2 cells, circadian proteins clearly regulate Th17 cell differentiation (85). Deficiency of RORα or RORγ impairs Th17 development in mice. Consistent with this, mice maintained under chronic light cycle perturbations exhibit altered Th17 cell frequencies in the intestines relative to mice maintained under a normal light cycle. Since Th17 cells have been implicated in neutrophilic asthma (86), clock disruption may affect specific phenotypes of asthma.

Regulatory T cells (Tregs) contribute to prevention of allergic disease by regulating effector cells. In humans, genetic deficiency of Tregs shows allergic manifestations (87). Interestingly, mice subjected to perturbed light/dark cycles (6-h advance every 4 days) have fewer Tregs in the intestine than mice subjected to normal light/dark cycles; this is associated with the development of food allergy (88). Consistently, nurses with regular day/night-shift rotation exhibit an increased incidence of food allergy in comparison to nurses with no such rotation of work hours (88). Thus, clock disruption may decrease the number of Tregs, thereby precipitating allergic disease.

B-Cell Response

The connection between clock and B cell response is not well-studied. Bmal1-deficient mice have fewer pre-B cells in the bone marrow, as well as fewer B cells in the peripheral blood and spleen (89). This likely involves Bmal1 deficiency in the bone marrow microenvironment, but not B cell–intrinsic Bmal1 (89, 90). Thus, Bmal1 may play a role in the normal differentiation of B cells, but its relevance to allergy remains unknown.

Collectively, these findings suggest that the circadian clock is strongly linked to two fundamental biological aspects of allergic disease, epithelial barrier function and immune responses (**Figure 1**).

ALLERGIC IMMUNE RESPONSE MAY TEMPORALLY COMPLEMENT EPITHELIAL BARRIER FUNCTION

Circadian gating of epithelial barrier function and immune response likely evolved to anticipate environmental physical, chemical, and biological insults (e.g., hot air, pollutants, pathogens) and to maximize host defense during the greatest time of the insults' exposure (11, 13). The temporal gating can also limit the costs of the defense response and may increase host fitness (11, 13).

Allergic immune response, in particular IgE/mast cell-mediated response, is thought to evolve to confer protection against macroparasites such as helminth worms and biting arthropods such as mite and mosquito (91). Therefore, it is possible that the circadian control of immune responses associated with allergic disease deal with helminth and biting arthropods that themselves behave in a circadian manner (92, 93). We speculate that allergic immune response may temporally complement epithelial barrier function (**Figure 2**). For instance, skin, intestinal, and possibly airway barrier functions largely weaken in the resting phase (34, 40). This may be due to less exposure to noxious stimuli such as hot air and food-borne bacteria and fungi associated with feeding behavior in the resting phase than in the active phase. On the other hand, clock system may maximize mast cell response to IgE during the resting phase (14, 15) in order to prepare for the risk of attack by helminth and biting arthropods at that time. In other words, helminth and biting arthropods might evolve to attack target animals at the time-of-day when the animals' epithelial barrier function is weakest.

CONCLUDING REMARKS: CIRCADIAN-DISRUPTED MODERN LIFESTYLES MAY PROMOTE ALLERGIC DISEASE

The periodicity of allergic disease impacts on diagnostic and preventive aspects as well as on treatment effects. Recent studies highlight that circadian clock underpins allergic reaction, which likely confers the periodicity of allergic disease (14–16). Importantly, it is becoming clear that the circadian clock is a potent regulator of allergic reaction with more than a simple time-keeping role (16). Of note, several studies suggest that the circadian clock is strongly linked to two fundamental biological aspects of allergic disease, epithelial barrier function and immune responses. Thus, clock disruption can precipitate and enhance disease by deregulating the epithelial barrier and immune functions.

This new knowledge highlights circadian disruption as a new precipitating factor of allergic disease in modern society, in which our sleep, work, and eating habits are out of sync with endogenous circadian rhythmicity. This may partly explain why allergic disease is so prevalent in developed countries. Accordingly, the relationship between circadian biology and allergy will become an important area of research to understand allergic diseases in the modern era and exploring new ways to prevent or treat these disorders. In this context, we propose that lifestyle or therapeutic interventions that align the endogenous circadian clock with environmental cycles should be a part of the efforts to prevent or treat allergic disease in 24/7 society.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was supported in part by JSPS KAKEN Grant Number 18H02848 and 19K22636 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ACKNOWLEDGMENTS

The author thanks all the members of our laboratory and Ms. Yukino Fukasawa for her valuable general assistance. Regrettably, due to space limitations, I was unable to refer to many important papers in this field.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 23 June 2020
doi: 10.3389/fimmu.2020.01235

Adrenergic Signaling in Circadian Control of Immunity

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Circadian rhythms govern a multitude of physiologic processes, both on a cell-intrinsic level and systemically, through the coordinated function of multi-organ biosystems. One such system—the adrenergic system—relies on the catecholamine neurotransmitters, adrenaline and noradrenaline, to carry out a range of biological functions. Production of these catecholamines is under dual regulation by both neural components of the sympathetic nervous system and hormonal mechanisms involving the hypothalamus-pituitary-adrenal axis. Importantly, both neural and hormonal arms receive input from the body's central clock, giving rise to the observed rhythmic variations in catecholamine levels in blood and peripheral tissues. Oscillations in catecholamine signals have the potential to influence various cellular targets expressing adrenergic receptors, including cells of the immune system. This review will focus on ways in which the body's central master clock regulates the adrenergic system to generate circadian rhythms in adrenaline and noradrenaline, and will summarize the existing literature linking circadian control of the adrenergic system to immunologic outcomes. A better understanding of the complex, multi-system pathways involved in the control of adrenergic signals may provide immunologists with new insight into mechanisms of immune regulation and precipitate the discovery of new therapeutics.

Keywords: sympathetic nervous system, circadian rhythms, adrenergic, immunity, noradrenaline

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Hendrik B. Sager, Deutsches Herzzentrum München, Germany
Henrik Oster, University of Lübeck, Germany

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *07 April 2020*
Accepted: *18 May 2020*
Published: *23 June 2020*

Citation:
Leach S and Suzuki K (2020) Adrenergic Signaling in Circadian Control of Immunity. Front. Immunol. 11:1235. doi: 10.3389/fimmu.2020.01235

CIRCADIAN RHYTHMS AND THE ADRENERGIC SYSTEM

Circadian Rhythms

Circadian rhythms are biological processes that exhibit intrinsic, self-sustained oscillations even in the absence of external, environmental cues. In mammals, these processes are maintained at the cellular level by transcriptional feedback loops that regulate expression of biological clock genes in a rhythmic fashion, following an approximately 24-h cycle. These cell-autonomous clocks are synchronized to function in concert at the bio-systems level, giving rise to the cyclic rhythms observed in, for instance, cardiovascular and endocrine function. Circadian rhythms are entrained by external signals (or "zeitgebers") such as light/dark cycles, temperature, sleep and feeding patterns, which, although not required for the maintenance of endogenous oscillations, function to synchronize the biological clock with the surrounding environment. Circadian rhythms are controlled by a central, light-sensitive "master clock"—the suprachiasmatic nucleus (SCN)—that coordinates the function of peripheral, tissue-regulated- and cell-autonomous-clocks (1). This hierarchical organization supports the synchronization of multi-organ systems while simultaneously maintaining the ability to independently fine-tune or uncouple local responses to circadian signals.

The SCN is located in the hypothalamus directly above the optic chiasm where it receives input from photosensitive ganglion cells in the retina. Photic signals are transmitted by neural fibers from the retina along the retinohypothalamic tract (RHT), eventually initiating a neurotransmitter-driven signaling cascade involving glutamate, PACAP (pituitary adenylate cyclase-activating polypeptide) and aspartate (2, 3). The RHT terminates at the ventrolateral region of the SCN, forming direct contact with SCN neurons in this region (4, 5). Here, neurotransmitter signaling through NMDA (N-methyl-D-aspartate) or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) receptors results in CREB binding to cAMP response elements in promoters of the so-called "clock genes" (6–9). Although this CREB-mediated regulation of clock gene expression is by far the most well-characterized mechanism linking circadian transcriptional feedback loops with photic signals, the search for alternative mechanisms is an area of active investigation (10). Thus, light cues are thought to be integrated into the molecular pathways governing circadian rhythmicity, providing a basis for photic entrainment of various biorhythms.

On a cellular level, control of circadian rhythmicity is thought to involve the interactions of at least two major autoregulatory transcription-translation feedback loops. The central players in the first feedback loop are the CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1) proteins (11, 12). CLOCK and BMAL1 interact to form a heterodimer which drives expression of PER (period circadian protein) and CRY (cryptochrome) proteins by binding to E-boxes in the *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* promoters (13, 14). PER and CRY, conversely, act as transcriptional repressors by displacing CLOCK-BMAL1 from E-box regulatory elements (15). The second feedback loop involves the nuclear receptors REV-ERBα, REV-ERBβ, and RORα (retinoic acid receptor-related orphan receptor alpha) (16–18). REV-ERBα and REV-ERBβ themselves undergo cyclic, circadian expression under the transcriptional activation of CLOCK-BMAL1 and repression by CRY-PER, while also exhibiting repressive control of CLOCK and BMAL1 expression (18). RORα, on the other hand, competes with REV-ERBα to drive BMAL1 expression (19). Together, these interlocking, auto-regulatory transcription-translation loops constitute the molecular basis for the cyclic gene expression driving circadian biorhythms. More extensive reviews of the molecular mechanisms underlying circadian rhythmicity can be found elsewhere (20–22).

The Adrenergic System

The adrenergic system is a neuro-hormonal system that regulates a range of physiological functions which are carried out through production of the catecholamines, adrenaline (epinephrine; EP) and noradrenaline (norepinephrine; NE). EP and NE signal through adrenergic receptors expressed on a wide variety of tissues and cell types, and are involved in processes such as regulation of cardiac function (23, 24), vascular remodeling and fat metabolism (25, 26), smooth-muscle-mediated vaso- and broncho-constriction (27), placental development (28), and control of immune function (29–31). Catecholamine production is regulated systemically via humoral messengers generated by the hypothalamus-pituitary-adrenal (HPA) axis, and locally by neural components of the sympathetic division of the autonomic nervous system. EP and NE are synthesized at a 4–1 ratio (favoring EP) (32) in the adrenal medulla and released into the bloodstream to carry out systemic functions. Neurons of the sympathetic nervous system (SNS), on the other hand, produce and predominantly secrete NE at discrete locations marked by the presence of adrenergic nerve terminals, thereby supplying peripheral tissues with highly localized NE signals. Importantly, the adrenergic system is one of the many biological systems thought to be under circadian control.

Rhythmic Catecholamine Production

In 1943 Pincus (33) made the preliminary observation that the concentration of certain adrenal hormones in urine oscillated following a night-day pattern. Two decades later, isolated adrenal glands were found to exhibit intrinsic metabolic rhythmicity in culture, pointing to the existence of a self-sustained, endogenous clock (34). Following this discovery, a role for the SCN as a regulator of circadian adrenal function was suggested by ablation of circadian oscillations in adrenal corticosterone content following lesioning of the SCN (35). Consistent with these reports detailing both endogenous and exogenous control of circadian fluctuations in adrenal function, diurnal rhythms in plasma EP and NE levels were also described. Humans were found to have low circulating catecholamine levels during the night and high levels during the day (36), while rodents exhibited the opposite pattern (corresponding to opposite periods of activity) (37).

However, although EP and NE exhibited, overall, similar 24-h rhythms in circulation, many early studies reported differences in the maintenance of EP and NE oscillations under free-running conditions (or in the absence of entrainment). Specifically, EP was reported to exhibit clear, self-sustained rhythmicity, while NE levels were found to adjust rapidly to sleep/wake patterns, leading many to conclude that rhythmicity in circulating NE levels was just a result of sleep or even postural cues (36, 38–40). Later studies more clearly demonstrated this distinction by showing that NE cycles were abolished under constant light or food-deprivation conditions (40, 41). These findings led to the conclusion that while oscillations in circulating EP appear to be circadian and are regulated by the HPA axis, cyclic variations in circulating NE exist only in the presence of cyclic, external zeitgebers and, therefore, cannot be considered truly circadian according to the strictest definition.

There is evidence, however, that the release of NE from sympathetic neurons within tissues is under circadian control. This was pointed to, for example, by the finding that NE in cerebrospinal fluid (CSF) [which is likely neuron-derived, as NE does not readily pass the blood-CSF barrier (42)] exhibits a circadian rhythmicity that is maintained despite disruption in light cycles (43). In addition, NE turnover in the pineal gland was demonstrated to exhibit an endogenous, 24-h rhythmicity that was maintained in blinded rats. The authors concluded that NE turnover in the pineal gland likely reflected daily rhythms in NE

release from sympathetic neurons within this tissue (44). As a related side note, neuron-derived NE signals activate pinealocytes in the pineal gland through β2-adrenergic receptors, driving circadian oscillations in the production of melatonin from its metabolic precursor, serotonin (44, 45). Melatonin has been demonstrated to exhibit a range of effects on immunity including control of cytokine production by neutrophils and lymphocytes (46, 47), inhibition of nitric oxide synthesis (48) and promotion of immune cell migration (49, 50), to name a few examples. Thus, circadian NE rhythms feed into rhythmic variations in indolamine metabolism, providing another, albeit indirect, way for the noradrenergic system to influence immune responses.

Regulation of Systemic Catecholamine Production by the HPA Axis

Release of EP and NE into circulation has long been thought to be regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Consistent with the observation that circulating catecholamine levels (particularly EP) exhibit circadian rhythmicity, neuroanatomical tracing studies demonstrated that SCN neurons of the circadian master clock are directly linked to the PVN (paraventricular nucleus neurons) of the hypothalamus—often considered the driver of the HPA axis (51). While the specific mechanisms by which signals governing circadian rhythmicity are propagated remain incompletely defined, various neurotransmitters have been proposed to be involved in SCN-PVN communication including vasopressin (52), vasoactive intestinal peptide (VIP) (53, 54) and neuromedin U (55). Moreover, the hypothalamus is known to release corticotropin-releasing hormone (CRH) and vasopressin in circadian fashion (52, 56). These PVN-derived peptides regulate pituitary function and drive the secretion of adrenocorticotropic hormone (ACTH), which then incites the adrenal cortex to produce glucocorticoids (cortisol in humans; corticosterone in rodents). Within the adrenal gland, glucocorticoids are transported to the medulla where chromaffin cells produce NE. Here, glucocorticoids activate the enzyme, phenylethanolamine *N*-methyltransferase (PNMT), which is required for the conversion of NE to EP (57, 58). Thus, cyclic variation in HPA signals are likely particularly important in driving diurnal oscillations in adrenal EP levels. Although our understanding of these processes remains far from complete, signals from the body's central, master clock are, in this way, thought to be translated into rhythmic variations in neurotransmitters and hormonal messengers, which influence peripheral sites in circadian fashion. This includes the cyclic release of catecholamines by the adrenal gland into the bloodstream.

Regulation of Systemic Catecholamine Production by the Sympathetic Adrenal Medullary Axis (SAM)

In addition to HPA-mediated regulation (and of arguably greater physiological importance for the production of adrenal catecholamines), adrenal glands are also innervated by neurons connected (via a polysynaptic pathway) to the SCN, providing a mechanism for direct regulation of adrenal catecholamine production by the body's master clock (59). Innervation of the adrenal medulla by preganglionic sympathetic neurons emanating directly from the spinal cord (60, 61), as well as splanchnic (62) and vagus nerves (63, 64), has also been reported. Neural stimulation of chromaffin cells and subsequent catecholamine secretion is thought to be induced by cholinergic signals, much in the same way that post-ganglionic, sympathetic neurons are activated to secrete NE by pre-ganglionic neurons in sympathetic ganglia (65, 66).

Consistent with these anatomical studies demonstrating neural connections between adrenal glands and the SCN, adrenal clock gene expression has been shown to be responsive to light entrainment (67). Furthermore, light-induced, acute activation of adrenal nerves is abolished in SCN-lesioned mice (68). This, combined with the observation that the rhythmicity in adrenal clock gene expression is unaltered in hypophysectomized rodents (69), lends support to the notion that adrenal glands may be subject to circadian oversight via neural, as well as hormonal, pathways. Thus, while HPA axis-mediated stimulation of chromaffin cells influences the ratio of EP to NE levels (70) in a cyclic fashion via glucocorticoid-driven PNMT activation (71), adrenal secretion of catecholamines also involves a local neural component—importantly, one that displays circadian rhythmicity (72) and is directly entrained by environmental cues organized in the SCN (68, 69). Although both the HPA axis and SAM axis potentially contribute to the oscillations in circulating catecholamine levels, the relative importance of these pathways under physiological conditions is not entirely clear. However, it has been suggested that the effects of the neuronal pathway may be manifest more prominently during prolonged or chronic disease states in which adrenal output and pituitary output do not correlate well (73–75) (**Figure 1**).

Tissue-Localized Catecholamine Production by Sympathetic Neurons

Sympathetic neurons innervate, in addition to adrenal glands, a wide range of tissues including primary and secondary lymphoid organs (76, 77). The primary neurotransmitter thought to be produced by SNS nerve terminals is NE (78). In circulation, NE concentrations are reported to be in the picomolar to low nanomolar range (79), but under conditions of strong sympathetic stimulation, the tissue concentration at nerve endings may reach micromolar levels (80). Given the relatively low amount of NE in the blood, one might question whether circulating NE has a significant physiological role at steady state or under healthy conditions. Indeed, urinary concentrations of NE remain constant after adrenalectomy in humans (81) suggesting that neuron-derived NE in peripheral tissues may be more physiologically relevant for NE-specific functions than circulating NE. In regards to immune function, the observation that lymphoid tissue exhibits diurnal fluctuations in NE is particularly noteworthy, pointing to possible circadian control of SNS neuronal NE output (82).

Adrenergic Receptors and Their Ligands

Catecholamines signal through adrenergic receptors, a class of G protein-coupled receptors. Broadly classified as α- or

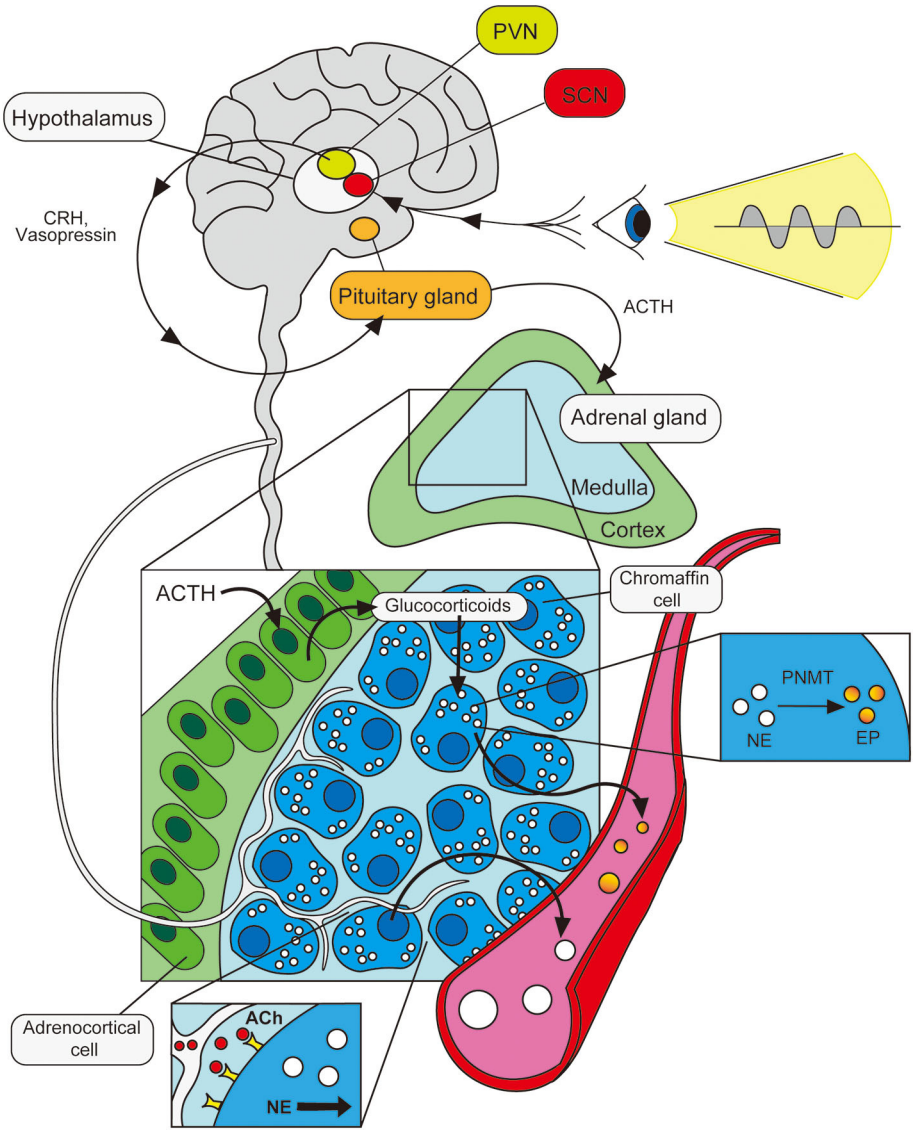

FIGURE 1 | Circadian control of adrenal catecholamine output. Photic signals entrain the body's master clock in the hypothalamus (SCN), sending oscillating signals to the PVN. The PVN propagates these neural signals to the pituitary gland via various neurotransmitters and hormonal messengers. The pituitary gland responds by releasing ACTH, which drives adrenocortical cells in the adrenal gland to produce glucocorticoids. These signal to chromaffin cells in the medulla, activating the enzyme, PNMT, which catalyzes the conversion of NE stores into EP for release into the bloodstream. The adrenal medulla is also innervated by acetylcholine (ACh)-producing neurons which also respond to cyclic light signals in the SCN. Stimulation of these neurons activates chromaffin cells, driving the release of NE into the bloodstream in a cyclic fashion.

β-adrenergic receptors (α-ARs, β-ARs), α-ARs exhibit higher affinity for NE whereas β-ARs exhibit higher affinity for EP (83, 84) (although neither type of receptor exhibits exclusive binding to NE or EP). These two classes are further subdivided into three α1- (α_{1A}, α_{1B} and α_{1D}), three α2- (α_{2A}, α_{2B} and α_{2C}), and three β- (β1, β2 and β3) adrenergic receptor subtypes (85–88) which display varied tissue-tropism and activate distinct signaling pathways. While varying degrees of expression of all three AR types have been reported on different hematopoietic cells, β2-ARs are thought to be predominantly expressed (31) [albeit with a notable absence on both resting and activated type 2 helper T cells (89, 90)]. Examining expression of different receptor subtypes is hindered by lack of specific antibodies and receptor subtype-specific ligands. As a result, current understanding of receptor expression comes largely from transcript-level analyses or competitive binding/saturation studies; more comprehensive studies are still needed in this regard.

Catecholamine Signaling Pathways

Signaling through adrenergic receptors reportedly modulates various cellular functions or properties, including (but not limited to) cell cycle and proliferation (91, 92), migration (93),

and cytokine (94) and antibody (95) production. Although historically, signaling through β-ARs has often been associated with anti-inflammatory outcomes, in more recent years, the ability of β-ARs to mediate context-dependent, pro- and anti-inflammatory effects has become more widely appreciated (29, 94, 96). β-ARs signal through the G-protein, $G\alpha_s$. Activation of $G\alpha_s$ leads to activation of adenylate cyclase (AC), which catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Subsequently, cAMP activates protein kinase A (PKA), which is responsible for the activation of downstream transcription factors. Although all β-ARs signal through $G\alpha_s$, it is thought that receptor subtype-specific differences in signal transduction are a result of specific localization of, for instance, membrane or cytosolic AC and PKA isoforms which form unique signalosomes, leading to the compartmentalization of cAMP pools which can then selectively target distinct physiological pathways (97–99). Non-canonical, G-protein-independent signaling through β-ARs has also been described (involving G protein-coupled kinases (GRKs) and β-arrestins), leading to the activation of ERK1/2 or MAPK pathways (100, 101). Intracellular cAMP levels have also been reported to influence, among other things, transcription of various cytokines in immune cells. For instance, increased cAMP leads to upregulation of IL-10 gene expression (102) while inhibiting IL-12 and TNF-α transcription (102, 103).

Much less is known regarding α-AR signaling on immune cells. Although not an exhaustive list, expression of α1-ARs has been reported on murine HSC progenitors (104), immature dendritic cells (93), mast cells (105), and human NK cells (106), while α2-AR expression has been reported on Kupffer cells (107), dendritic cells (108), NK cells (106), and occasionally on human lymphocytes (109, 110). Most of our current knowledge regarding signal transduction comes from studies of cardiomyocytes in which α1-ARs are coupled to $G\alpha_q$ G-proteins. Ligand binding then leads to the activation of PLCβ1, downstream calcium signaling and activation of PKC (111). α2-ARs, on the other hand, associate with G_i-type G proteins, and drive inhibition of AC and reduction of cAMP levels upon ligand binding (112, 113), potentially providing a means to counteract the effects of β-AR stimulation.

CATECHOLAMINES IN THE CENTRAL NERVOUS SYSTEM AND DISEASE

Catecholamine Production in the Central Nervous System

The locus coeruleus (LC) is the primary source of NE in the central nervous system, sending projections throughout the brain (114). It is thought to have a role in attention and arousal (115), as well as in stress-responses such as fear or anxiety (116, 117). Moreover, retrograde tracing experiments have revealed trans-synaptic circuits connecting the SCN and LC, and providing a basis for circadian control of LC activity (118, 119). In addition to circadian effects on LC firing, however, LC-derived NE levels both influence and are heavily influenced by sleep state (being completely absent during REM sleep) and changes in vigilance or alertness (120, 121), making rhythms in brain NE levels quite sensitive to fluctuation. This difficulty in assessing the specific circadian contribution to NE oscillations in brain tissue has proven a challenge to studies that might attempt to directly link circadian control of central catecholamine production and immune function. Nevertheless, indirect effects of LC-derived NE on immunity are expected to exist, for instance, through modulation of sleep and its general impact on whole body function.

Catecholamines in the Central Nervous System and Immunity

The blood-brain barrier hinders direct interactions between LC-derived NE and most immune cell subsets. However, microglia—the brain's resident innate immune cells—are known to be highly sensitive to catecholamine signals, expressing higher levels of β2-adrenergic receptors than any other cell type in the brain (122, 123). Studies in mice have demonstrated an inhibitory role for LC-derived NE on microglial function involving changes in activation, nitric oxide production, tissue surveillance and gross morphology (124–126). Thus, one may easily imagine that circadian oscillations in NE coordinate microglia-neuron dynamics to promote homeostasis under normal physiological conditions, and that perturbations in adrenergic signaling might be particularly deleterious (or perhaps even a causative factor) in various neurological disease states. Indeed, LC dysfunction or degeneration is widely observed at early stages in both Alzheimer's disease and Parkinson's disease patients (127–129).

CIRCADIAN CONTROL OF IMMUNE CELL TRAFFICKING MEDIATED BY ADRENERGIC SIGNALS

Diurnal Variation in Leukocyte Trafficking Patterns

The most well-characterized means through which adrenergic signals exert circadian control over immunity is by regulation of cell trafficking. Diurnal variation in the number of blood circulating leukocytes was reported as early as the 1940s in both mice and humans (130, 131), with most reports describing peak blood leukocyte numbers during the inactive period. Some discrepancies exist, however, in the literature regarding cell-type-specific, day vs. night circulation patterns (130, 132–136). Nevertheless, this phenomenon seemed to be linked to adrenergic function as administration of adrenal or pituitary hormones resulted in blood lymphopenia (137) and adrenalectomy abolished cyclic variations in circulating leukocyte numbers (133, 138). As these early studies all pointed to a role for systemic (endocrine) catecholamine-mediated regulation of cyclic variation in leukocyte circulation, an important development in our understanding of this process was the finding that tissue-localized catecholamine signals originating from sympathetic nerve terminals could also significantly influence diurnal patterns in leukocyte migration between blood and peripheral tissues.

Adrenergic Signaling in Stromal Cells Controls Immune Cell Egress From BM

Hematopoietic stem and progenitor cells (HSPCs) are constitutively released from BM into the circulation at steady state. However, mobilization of hematopoietic progenitors is dramatically increased under inflammatory conditions or "emergency" states in anticipation of increased requirements for hematopoietic output. Expression of CXCL12 within bone tissue is known to be important for retention of $CXCR4^+$ HSPCs in hematopoietic niches and suppression of their mobilization. Conversely, granulocyte colony-stimulating factor (G-CSF) has long been recognized for its strong, mobilization-promoting properties. While exploring the mechanisms underlying G-CSF-induced HSPC mobilization, Katayama et al. made the somewhat surprising observation that destruction of peripheral adrenergic neurons by chemical sympathectomy (6-OHDA-(6-hydroxydopamine) administration), or treatment of mice with a β-AR antagonist led to a significant reduction in G-CSF-dependent, HSPC mobilization (139). This pointed to a role for neuron-derived adrenergic signals in this phenomenon. However, β2-AR agonist stimulation alone did not promote mobilization, suggesting that G-CSF and adrenergic signals somehow functioned cooperatively in this process. The authors also demonstrated a correlation between decreased osteoblast CXCL12 expression and HSPC mobilization. In conclusion they proposed that G-CSF suppresses CXCL12 production by osteoblasts, thereby promoting HSPC mobilization. In parallel, G-CSF and sympathetic neuron-derived NE signals were suggested to contribute to this process by some undefined mechanism. Although this initial study simply pointed to a role for adrenergic signals in HSPC mobilization, several subsequent studies have since sought to address the mechanistic questions raised by these observations.

In 2008, Méndez-Ferrer et al. demonstrated that the diurnal variation in HSPC trafficking between BM and blood is a true, circadian phenomenon, as the observed rhythmicity was maintained under constant dark conditions and was abolished in *Bmal1*-deficient mice (140). The authors also confirmed, via surgical sympathectomy of the hind leg, that local, neuron-derived adrenergic signals in the BM were required for this process. Similar to the report by Katayama et. al., downregulation of CXCL12 expression was proposed to be the mechanism driving HSPC egress. However, in contrast to previous reports, Méndez-Ferrer and colleagues demonstrated that adrenergic signals were detected by β3-ARs (rather than β2-ARs) on some non-osteoblast, BM stromal subset [later proposed to be $nestin^+$ mesenchymal stem cells (141)]. Moreover, stimulation of β-ARs with a non-subtype-specific β-agonist (isoprenaline) alone was sufficient to promote HSPC egress (**Figure 2**). This study provided intriguing evidence that circadian control of adrenergic signals may be discretely regulated in peripheral anatomic locations (such as the BM) by the sympathetic nervous system. The finding that localized, surgical sympathectomy could abolish circadian oscillations in HSPC output from BM, however,

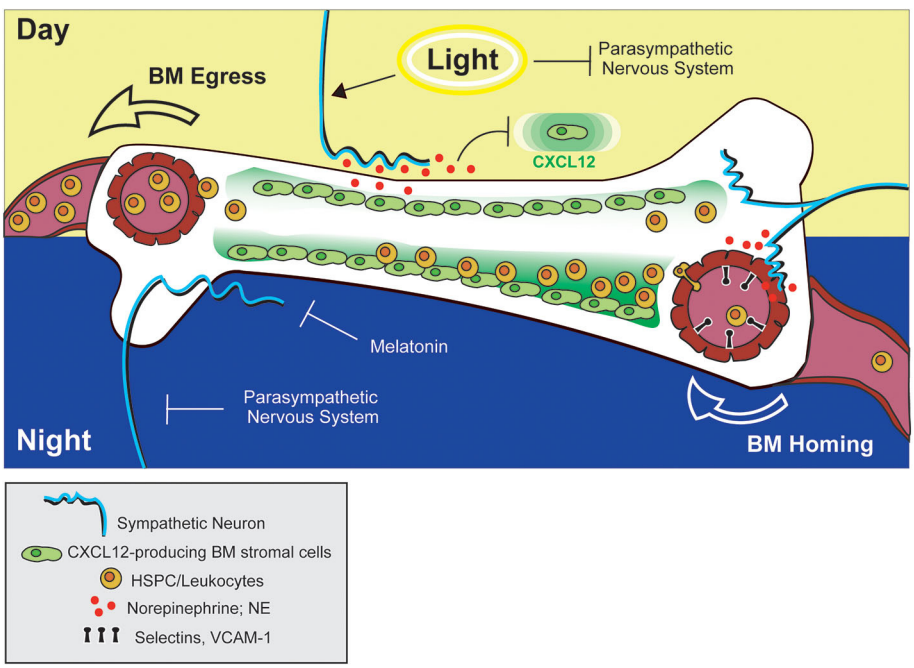

FIGURE 2 | Adrenergic control of leukocyte and HSPC trafficking to and from BM. Circadian stimulation of sympathetic neurons drives down-regulation of CXCL12 in the BM, facilitating HSPC egress into circulation during the day (in mice). At night, adrenergic signals from sympathetic neurons promote upregulation of adhesion molecules by blood endothelial cells in bone tissue, promoting BM homing of leukocytes. The combined actions of melatonin and the parasympathetic nervous system may also function to prevent down-regulation of CXCL12 (and BM egress) during this time.

seems at odds with early studies reporting similar findings after adrenalectomy and raises questions regarding the relative importance of tissue-localized vs. systemic adrenergic signals in driving BM egress of immune cells. To our knowledge, a side-by-side comparison of the effects of adrenalectomy and surgical sympathectomy on circulating immune cell subsets has not been carried out, but would help shed some light on this issue.

Adrenergic Signaling Mediates Immune Cell Recruitment to Peripheral Tissues

The concept that adrenergic signals could influence circadian variations in HSPC circulation was later expanded by studies examining the effects of adrenergic signaling on immune cell trafficking to peripheral tissues at rest or under inflammatory conditions. Scheiermann et al. reported that variation in recruitment of leukocytes from blood to BM or skeletal muscle also exhibited diurnal tendencies, with rhythms oscillating in antiphase to that observed for HSPCs traveling from BM to blood (142). This phenomenon exhibited genuine, circadian characteristics as shown by ablation of oscillatory patterns in *Bmal1*-deficient mice (which lack a core component of the molecular circadian clock) and, conversely, maintenance of these patterns in the absence of light. The cyclic recruitment of leukocytes to peripheral tissues coincided with cyclic variations in expression of different tissue-specific adhesion molecules and chemokines expressed by endothelial cells at the site of extravasation. Diurnal variation in ICAM-1 and CCL2 was observed in skeletal muscle endothelial cells, while expression of P-selectin, E-selectin and VCAM-1 fluctuated within BM endothelium (**Figures 2**, **3**). Importantly, surgical sympathectomy to denervate the BM or cremaster muscle abolished both cyclic leukocyte recruitment and oscillations in adhesion molecule expression, suggesting that, similar to their role in HSPC egress from BM, adrenergic nerves may provide tissue-specific signals that drive local changes in expression of guidance cues, thereby instructing immune cell trafficking in a diurnal fashion corresponding to the circadian nature of the adrenergic signals they provide. Adoptive transfer studies using receptor knock-out mice pointed to a role for both β2- and β3-ARs in this process (although only a requirement for β3-AR signaling could be confirmed using a similar agonist-based approach). Finally, the cyclic recruitment of leukocytes to peripheral tissues was shown to influence the pathological severity of various types of inflammatory challenge in disease models including septic shock and sickle cell vaso-occlusion (142).

One major question raised by these findings was how temporal changes in BM egress and recruitment could sustain overall cyclic patterns in immune cell circulation when both processes were reported to be governed by the same signals. While Scheiermann et al. reported that adrenergic nerves signal through β3-ARs on BM endothelium driving increased expression of selectins and VCAM-1 to promote immune cell recruitment to BM (142), Méndez-Ferrer et al. reported that adrenergic nerves signal through β3-ARs in BM to promote downregulation of CXCL12 expression and HSPC egress (140). How could these seemingly opposing mechanisms function in concert?

Related to this apparent paradox, Golan et al. reported that HSPC numbers in BM exhibit, not one but two, peaks and troughs during a single day/night cycle—one at night, and one after morning light exposure (135). The authors proposed that the morning HSPC peak coincides with a transient increase in NE and TNF levels in the BM driving β2-AR-mediated HSPC proliferation and differentiation, which is followed by a wave of HSPC egress into the bloodstream mediated by β3-AR-dependent processes. In addition, a second, night-time peak in BM HSPC numbers overlaps with a second NE and TNF burst during which melatonin was shown to inhibit HSPC egress and differentiation (**Figure 2**). In the context of the previously mentioned reports, these two different HSPC BM peaks could conceivably be attributed to the alternate adrenergic mechanisms driving BM egress and recruitment. Under this interpretation, the light-induced HSPC peak could represent the NE and TNF-induced HSPC proliferative burst that is followed by NE-driven egress via mechanisms described by Méndez-Ferrer et al. (140), while the night-time peak might represent the effect of BM recruitment driven by NE signals as reported by Scheiermann et al. (142). Although this dual peak model provided a potential explanation for altered temporal discernment of these two adrenergic-driven processes in mice, in humans, light exposure coincides with the beginning of the active phase. In this case, it would be expected that the light-induced and active-phase NE peaks would at least partially overlap, making it unclear whether such a model is equally relevant for both diurnal and nocturnal species.

Recently another layer of complexity was introduced to account for the different temporal responses to adrenergic signals in BM by studies examining the additional contribution of the parasympathetic nervous system and cholinergic signaling in this process. Using *Gfra2* (GDNF family receptor α2)-deficient mice as a model of parasympathetic deficiency, García-García et al. showed that parasympathetic nervous system-derived cholinergic signals suppress excessive sympathetic noradrenergic activity (143). At night, this may function to prevent β3-AR-mediated HSPC egress, while permitting β2-AR-driven BM homing. During the day, however, inhibition of parasympathetic activity by light exposure permits the egress of HSPCs from BM via noradrenergic, β3-AR-mediated mechanisms (**Figure 2**). Supporting this model, the authors also reported diurnal oscillations in β2- and β3-AR expression in BM, with β2-AR higher at night, and β3-AR preferentially expressed during the day (143).

Immune Cell-Intrinsic Adrenergic Signaling

Initial studies examining the function of adrenergic signals in immune cell trafficking largely focused on the effects of catecholamines on non-hematopoietic cell types and their indirect effect on immune subsets. Nakai et al. brought further insight to the field with the finding that immune cell-intrinsic adrenergic signaling has the potential to alter a cell's migratory capability (144). This study began with the preliminary observation that treatment of mice with β2-AR agonists led to

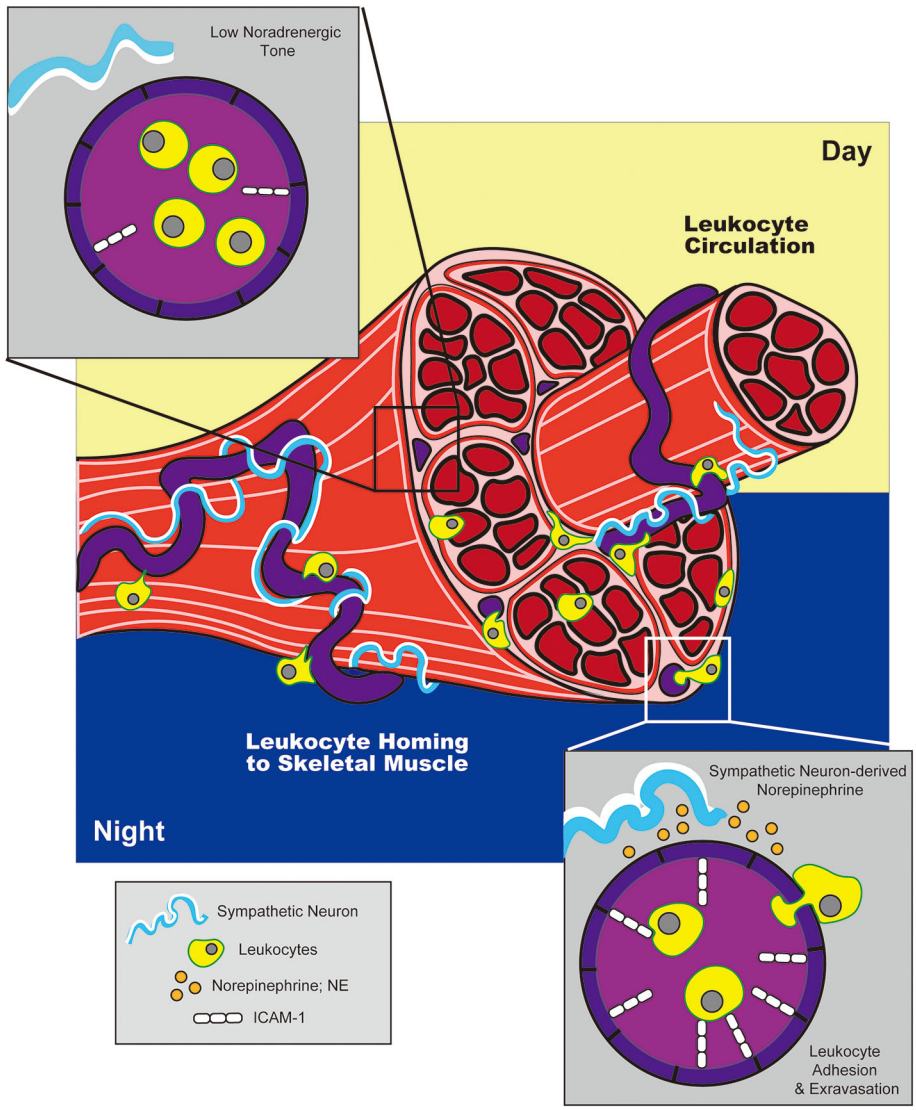

FIGURE 3 | Adrenergic control of circadian leukocyte trafficking to skeletal muscle. During the night (in mice), adrenergic signals from sympathetic neurons drive upregulation of adhesion molecule, ICAM-1, on blood endothelial cells in skeletal muscle. This promotes leukocyte trafficking to and extravasation into tissue during the night, while reduced ICAM-1 expression during the day facilitates return to circulation.

a rapid decrease in the number of circulating lymphocytes in both blood and lymph—a response that was largely lymphocyte-intrinsic, as later demonstrated using BM chimeras. Follow-up experiments showed that β2-AR stimulation effectively blocked lymphocyte egress from lymph nodes (LNs) leading to the concomitant reduction of lymphocyte numbers in circulation. Notably, while B cells, $CD4^+$ and $CD8^+$ T cells all responded to adrenergic stimulation, the effect was most pronounced in the B cell compartment, likely as a result of higher intrinsic β2-AR expression within this population. Moreover, LN-innervating sympathetic neurons were proposed to be the source of these adrenergic signals as 6-OHDA treatment led to a reduction in the number of lymphocytes retained in entry-blocked LNs (treated with integrin-neutralizing antibodies) compared to those in innervated controls (It should be noted, however, that the contribution from adrenal gland-derived catecholamines cannot be formally excluded). Prolonged β2-AR stimulation was shown to have a clear physiological impact on the severity of inflammatory T cell-driven responses in both the mouse model of multiple sclerosis, EAE, and delayed-type hypersensitivity (DTH) responses. These effects were mediated by the inhibition of T cell egress from LNs and, therefore, inhibition of migration to sites of disease progression (the central nervous system in EAE and the skin in DTH).

The mechanism by which adrenergic signals promote LN retention of lymphocytes was shown to involve increased sensitivity of retention-promoting chemokine receptors, CXCR4 and CCR7, for their respective ligands following co-stimulation

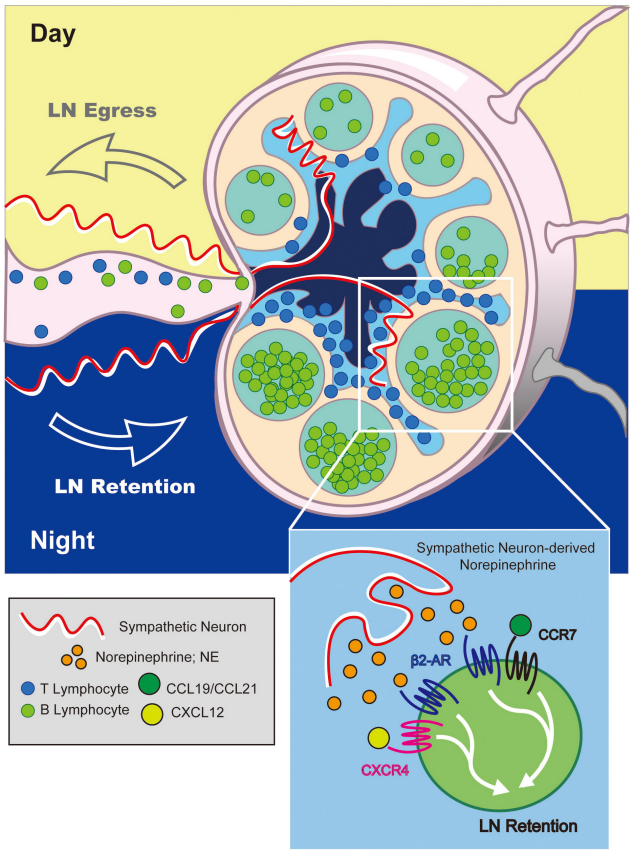

FIGURE 4 | Adrenergic control of diurnal LN egress and lymphocyte retention. At night (in mice), adrenergic signals from sympathetic neurons stimulate β2-ARs expressed on LN-resident lymphocytes. This increases the sensitivity of lymphocytes to CCR7 and CXCR4-mediated LN-retention signals inhibiting their migration back into lymph fluid. Conversely, reduction in adrenergic tone during the day promotes lymphocyte egress.

through the β2-AR (**Figure 4**). This was evidenced by prolonged Rac1 GTPase activation following co-stimulation, and was further supported by findings from membrane co-localization and immunoprecipitation experiments which pointed to potential physical interactions between β2-AR and CCR7 or CXCR4. Furthermore, *in vitro* migration assays demonstrated enhanced chemotactic responses in lymphocytes receiving dual β2-AR agonist and chemokine stimulation. Importantly, this mechanism was subsequently proposed to constitute the basis for diurnal oscillations in lymphocyte recirculation through lymph nodes in response to sympathetic neuron-derived signals (82).

Given that adrenergic signals are produced in a circadian manner from sympathetic nerve terminals, it followed that β2-AR-mediated control of lymphocyte retention in LNs might also contribute to the daily rhythmicity observed in circulating immune cells. In continuation of these previous studies (144), Suzuki et al. demonstrated that the diurnal fluctuations in blood- and lymph-circulating lymphocyte numbers varied in antiphase to that in LNs, with the active phase exhibiting high lymphocyte numbers in LNs, correlating with high LN adrenergic tone (82). These night/day fluctuations in LN lymphocyte numbers were abrogated after chemical denervation with 6-OHDA, suggesting that adrenergic nerves were providing the signals regulating cyclic LN egress. Moreover, the cyclic changes in lymphocyte numbers were dependent on lymphocyte-intrinsic β2-AR expression. Although not directly shown, the authors propose that this phenomenon likely also involved the enhanced sensitivity of lymphocytes to chemokine receptor stimulation under conditions of co-adrenergic stimulation as previously reported (144) (**Figure 4**). Finally, the authors demonstrated a very clear difference in the magnitude of the adaptive immune response elicited following immunization at night vs. during the day, with enhanced antibody, germinal center and T follicular helper cell generation evident after night-time immunization, when circadian NE signals in mice are known to be highest. Thus, this study clearly demonstrated that adrenergic signaling does not always lead to immuno-suppressive outcomes, but may, rather, support adaptive immunity to promote host defense in a context-dependent manner.

Apart from the reported role for adrenergic receptors in circadian lymphocyte trafficking through lymph nodes, others have reported that cell-intrinsic clocks may drive diurnal variation in lymphocyte numbers in secondary lymphoid organs (145). The role of cell-intrinsic clocks in lymphocyte function, however, has been somewhat disputed in recent years with some groups reporting no or limited effects (146) and others reporting, for instance, effects on lymphocyte differentiation (147). Given the multi-tiered organization of circadian control mechanisms, it may be argued that both central clock-mediated, adrenergic signaling and cell-intrinsic clocks may participate to an extent in this phenomenon. Although analysis of molecular, cell-intrinsic or central circadian clocks were not carried out in the study by Suzuki et al., sufficient evidence of circadian regulation of sympathetic neuron-derived NE signals exists to make a strong case for circadian control of lymphocyte egress from LNs as observed in this study (82).

In addition to these studies showing how lymphocyte-intrinsic adrenergic signaling may influence immune function, adrenergic signaling is likely to have varying cell-intrinsic effects on different hematopoietic subsets and is a topic of ongoing investigation. For instance, Spiegel et al. (148) reported that human $CD34^+$ HSPCs upregulate expression of β-ARs in response to G-CSF treatment. The authors also provided evidence for synergistic effects of G-CSF and adrenergic signaling (by EP) in HSPCs, including enhanced proliferative and repopulation potential after engraftment. These effects were mediated, at least in part, by activation of the Wnt-β-catenin signaling pathway. The finding that G-CSF modulates immune cell responsiveness to adrenergic signals provided an alternative explanation for how G-CSF signals function in concert with neuron-derived adrenergic signals to promote HSPC mobilization as originally noted by Katayama et al. (139).

CONCLUDING REMARKS

In this review we have attempted to give an overview of the ways in which the central circadian clock regulates rhythmic catecholamine synthesis and secretion, describe how catecholamines signal through adrenergic receptors in immune

cell targets, and highlight a few publications which incorporate all of these aspects in a demonstration of the physiological significance of circadian regulation of immunity through adrenergic signaling. With this background regarding the ways in which circadian clocks regulate adrenergic signals in mind, the interested reader is encouraged to survey the many reviews detailing how adrenergic signaling more broadly affects immunity. We anticipate that future studies examining the effects of adrenergic signaling on immunity may be benefitted from a broader understanding of adrenergic signaling as a process regulated by circadian mechanisms, with potential implications for modulation of immune outcomes.

AUTHOR CONTRIBUTIONS

SL and KS both wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Japan Society for the Promotion of Science (JP15H05656, JP15K15153, JP15H01157, and JP19H03489), the Japan Science and Technology Agency (PRESTO), the Japan Agency for Medical Research and Development (JP19gm6210007) and the Takeda Science Foundation to KS. SL was a recipient of the Kishimoto Foundation Fellowship.

ACKNOWLEDGMENTS

We thank the members of the Suzuki laboratory for helpful comments on the manuscript. We apologize to the many researchers whose work could not be recognized in this review due to space limitations.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 31 July 2020
doi: 10.3389/fimmu.2020.01630

Circadian Control of Inflammasome Pathways: Implications for Circadian Medicine

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The innate immune system senses "non-self" molecules derived from pathogens (PAMPs) as well as endogenous damage-associated molecular patterns (DAMPs) and promotes sterile inflammation that is necessary for injury resolution, tissue repair/regeneration, and homeostasis. The NOD-, LRR- and pyrin domain containing protein 3 (NLRP3) is an innate immune signaling complex whose assembly and activation can be triggered by various signals ranging from microbial molecules to ATP or the abnormal accumulation of crystals, thus leading to IL-1β and IL-18 maturation and secretion. Deregulation of the NLRP3 signaling cascade is associated with numerous inflammatory and metabolic diseases including rheumatoid arthritis, gout, atherosclerosis or type 2 diabetes. Interestingly, the circadian clock controls numerous inflammatory processes while clock disruption leads to or exacerbates inflammation. Recently, the biological clock was demonstrated to control NLRP3 expression and activation, thereby controlling IL-1β and IL-18 secretion in diverse tissues and immune cells, particularly macrophages. Circadian oscillations of NLRP3 signaling is lost in models of clock disruption, contributing to the development of peritonitis, hepatitis, or colitis. Sterile inflammation is also an important driver of atherosclerosis, and targeting the production of IL-1β has proven to be a promising approach for atherosclerosis management in humans. Interestingly, the extent of injury after fulminant hepatitis or myocardial infarction is time-of-day dependent under the control of the clock, and chronotherapy represents a promising approach for the management of pathologies involving deregulation of NLRP3 signaling.

Keywords: clock, inflammasome, NLRP3, Rev-erb, RORalpha, circadian immunity

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *14 April 2020*
Accepted: *18 June 2020*
Published: *31 July 2020*

Citation:
Pourcet B and Duez H (2020) Circadian Control of Inflammasome Pathways: Implications for Circadian Medicine. Front. Immunol. 11:1630. doi: 10.3389/fimmu.2020.01630

INTRODUCTION

Organisms evolved in presence of a recurring daily light–dark cycle generated by the rotation of the Earth. To adapt to this predictable environmental change, they developed an internal clock mechanism that is entrained to and anticipates environmental cues such as light or food availability and optimizes physiological functions by ascribing them to the best time window (1). Many, if not all, physiological pathways and functions are regulated in a daily manner including sleep/active alternance, metabolism, heart rate, brain and muscular activity, to cite a few. More recently, research efforts have been focused on the circadian behavior of the immune system that allows optimization of immune responses throughout the day/night cycle (2), leading to the emerging concept of circadian immunity.

As a consequence, alteration of the circadian clock aggravates acute and chronic inflammatory diseases, pointing to new pharmacological approaches (3, 4).

The NLRP3 inflammasome was identified as a critical immune component that orchestrates host immune homeostasis. However, its chronic activation by endogenous danger signals derived from tissue damage and abnormal accumulation of self-components including urea and hydroxyapatite crystals in joints, amyloid fibers in brain or cholesterol crystals in the vascular wall, contributes to the development of a wide variety of diseases (5). Hence, a tight control of its transcription and activation is required to avoid overt deleterious activation.

In this review, we summarize the current knowledge on clock-controlled inflammasome modulation and highlight the underlying mechanisms as well as gaps of knowledge. We discuss several pathological contexts in which clock alteration contributes to NLRP3-driven pathologies and the potential of a (re-) synchronization of the clock to fine-tune NLRP3 activation and restore tissue homeostasis.

INNATE IMMUNE SYSTEM AND PATTERN RECOGNITION RECEPTORS (PRRs)

The innate immune system is the first line of defense involved in the clearing of invaders like bacteria and viruses and also of abnormal accumulation of self-components including cellular debris or crystals. Immune cells discriminate infectious agents-derived molecules called pathogen-associated molecular patterns (PAMPs) and non-infectious, endogenous "danger molecules" or DAMPS (damage-associated molecular patterns) released by damaged or dying cells following tissue injury. These motifs are specifically recognized by tissue-resident cells such as mast cells, monocytes/macrophages, neutrophils and dendritic cells that express Pattern Recognition Receptors (PRRs). PRRs may be classified depending on their nature, their ligands and their cellular localization [see (6) for review]. Hence, they can be distinguished according to whether they are located at the cytoplasmic membrane (membrane PRRs: Toll-Like Receptors TLRs, C-type lectin receptors CLRs) or in the cytoplasm (cytoplasmic PRRs: NOD-Like Receptors NLRs, RIG-I-like Receptors RLRs and cytosolic DNA sensors CDSs). For instance, TLR-2 and TLR-4 are membrane receptors that are bound by PAMPs such as Gram+ peptidoglycans or Gram- LPS, respectively. Detection of PAMPs by PRRs triggers maturation and activation of immune cells that, in turn, secrete inflammatory factors and stimulate adaptive immunity (7). Non-infectious DAMPs are also recognized by PRRs on innate immune cells and initiate a so-called sterile inflammation. In addition to classical PRRs, numbers of non-PRR transmembrane proteins including Receptor for Advanced glycation endproducts (RAGEs), Triggering Receptors Expressed on Myeloid cells (TREMs), G Protein-Coupled Receptors (GPCRs) and ion channels are able to sense DAMPs and to trigger migration and activation of immune cells (6). PRRs and non-PRRs are involved in sterile inflammation and inflammatory diseases such as ischaemia-reperfusion injury, systemic lupus erythematosus, gout, neurodegenerative diseases, diabetes, colitis, atherosclerosis, hepatitis, rheumatoid arthritis, cancer, lung diseases, and gut diseases (6).

Inflammation is characterized by the production of histamine, cytokines, chemokines, and lipid derivatives (6). Cytokines are immunomodulatory signaling molecules playing a pivotal role in inflammation. The IL-1 cytokine family is composed of several members including IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ (7). Except for IL-1α, IL-1 cytokines are produced as inactive pro-cytokines and require maturation to biologically active forms by enzymatic cleavage. Among those, IL-1β is probably the most studied IL-1 family member because of its central involvement in acute and chronic inflammatory diseases. Pro-IL-1β, the inactive form of IL-1β, is processed by the proteolytic activity of Caspase 1, the predominant IL-1 processing protease. Caspase 1 activity is tightly controlled by cytosolic PRR-constituted inflammasome complex.

NOD-like receptors form the main class of cytosolic PRRs that are activated by diverse exogenous signals including anthrax lethal toxin (NLRP1), bacterial flagellin (NLRC4), double-stranded DNA Absent in Melanoma 2 (AIM2), Toxin-induced modifications of Rho-GTPase (Pyrin). In this regard, NLRP3 is unique because it acts as an intracellular innate immune sensor for a large variety of PAMPS and also DAMPs.

THE NLRP3 INFLAMMASOME: A STRESS SENSOR

The nucleotide-binding domain (NOD)-, Leucine-rich repeat (LRR)- and pyrin domain-containing protein 3 NLRP3 inflammasome was first identified in Cryopyrin-associated periodic syndrome (CAPS) before its implication was recognized in many inflammatory/immune diseases such as gout, atherosclerosis, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) (8), as well as neurodegenerative diseases (Alzheimer and Parkinson diseases) and aging (9–11), and infection by various pathogens (12). The NLRP3 inflammasome is mainly expressed by monocytes/macrophages, neutrophils and dendritic cells, but also by other cell types including hepatocytes (13), neurons (14), cardiomyocytes (15), pancreatic beta cells (16), or endothelial cells (17).

A Two-Step Activation Process

The NLRP3 inflammasome is a macromolecular protein complex whose assembly is hierarchically organized and mostly requires a sensor protein, an adapter protein and an effector protein. The NLRP3 protein is a sensor protein that is composed of a C-terminal leucine rich repeat (LRR) domain, a central oligomerization domain (NOD, nucleotide-binding and oligomerization domain, NACHT) and an N-terminal Pyrin effector domain (PYD). This last PYD interacts with the amino-terminal PYD domain of the apoptosis-associated speck-like protein containing a Caspase recruitment domain (ASC) protein to initiate the inflammasome assembly and the formation of the so-called ASC speck. ASC is playing the role of adapter platform for the Caspase 1 protein thanks to its a carboxy terminal CARD

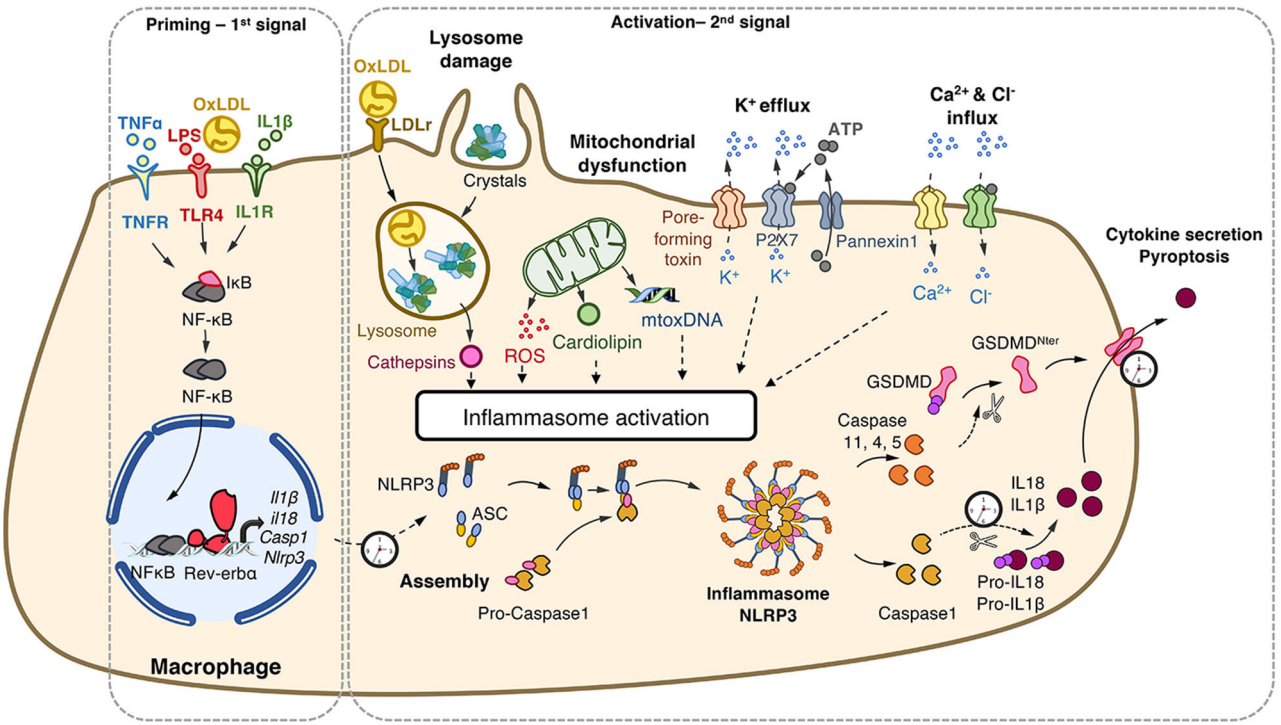

FIGURE 1 | NLRP3 inflammasome priming and activation. The priming (first step) of the NLRP3 inflammasome requires the binding and activation of PRRs (TLRs,...) by PAMPs such as LPS, cytokines or ox-LDL, resulting in the transcription of the NLRP3 inflammasome components. Its activation (second step) is the result of recognition of PAMPs (such as the bacterial pore-forming toxin nigericin) or DAMPs which are released by damaged or dying cells (such as ATP) following injury or metabolic imbalance (such as mtROS), or accumulate in tissues (such as crystals). These lead to lysosomal damage, mitochondrial damages (exposition of cardiolipin, mtDNA) which ultimately modify ion (K+, Ca2+) fluxes. Upon these two-step process, the NLRP3 inflammasome assembles, caspase-1 is activated, Gasdermin-D and pro-IL-1β and pro-IL-18 are cleaved, leading to mature cytokines secretion and cell death by pyroptosis. ASC, apoptosis-associated speck-like protein containing a CARD domain; ATP, adenosine triphosphate; casp, caspase; DAMPs, damage-associated molecular patterns; GSDMD, gasdermin-D; IL, interleukin; IL1R, interleukin-1 receptor; LDL, Low Density Lipoprotein; LDLr; LDL receptor; LPS, lipopolysaccharide; mtoxDNA, mitochondrial oxidized DNA; NFκB, nuclear factor-kappa B; NLRP3, nucleotide-binding, LRR and PYD domains-containing protein 3; Ox-LDL, oxidized low-density lipoproteins; PAMPs, pathogen-associated molecular patterns; PRRs, Pattern Recognition Receptors; ROS, reactive oxygen species; P2X7R, purinergic receptor P2X 7; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

domain that eventually recruits an unprocessed pro-caspase1 (18). Pro-caspase-1 oligomerization on the ASC filament enables proximity-driven autocatalytic caspase-1 maturation.

This complex activation is tightly controlled by a two-step process (**Figure 1**). A priming step is required to increase gene and protein expression of its components in order to sense stimuli and become activated (19). This priming occurs *via* ligand binding to PRRs (*eg.* signals that engage TLRs). These ligands may originate from exogenous sources such as bacterial wall components (Lipopolysaccharides, proteoglycans), or endogenous molecules (oxidized low-density lipoproteins [oxLDL], IL1, TNFα). This priming step is tightly controlled at the transcriptional level by the classical pro-inflammatory NF-κB and AP1 pathways, but also by metabolic sensors such as nuclear receptors including Liver X Receptors (20) and Rev-erb (21). A second step is the activation of the NLRP3 inflammasome in a primed cell/tissue that triggers the NLRP3 multimeric complex assembly that allows caspase 1 maturation and results in caspase1-mediated maturation of the pro-inflammatory interleukin-1β (IL-1β) and IL-18, the release of the mature cytokines, as well as in the so-called pyroptotic cell death (22). This second step may be triggered by a variety of compounds identifying NLRP3 as a wide PAMPs and DAMPs sensor as described below (8, 22, 23).

NLRP3: A PAMPs' and DAMPs' Sensor

The NLRP3 inflammasome detects a broad range of DAMPs and PAMPs. Cholesterol crystals that accumulate in the arterial wall during atherosclerosis (24), monosodium urate (MSU) accumulation in joints leading to gout (25) and hydroxyapatite crystals triggering rheumatoid arthritis (5) all activate the NLRP3 inflammasome (**Figure 1**). The internalization of crystals leads to lysosomal damage and subsequent cathepsins and Ca^{2+} release that activates NLRP3 in a yet unknown manner. In addition, NLRP3 activation is also triggered by metabolic stresses such as hyperglycemia, some fatty acids and ceramides, and mitochondrial dysfunction, in particular mtROS (26), exposition of cardiolipin (27) or presence of mitochondrial oxidized DNA (28). Bacterial pore-forming toxins such as nigericin act as ionophores promoting K^+ efflux which provokes the assembly of the NLRP3 complex, activation of Caspase 1 and the release of mature cytokines (29). Extracellular ATP released by dying

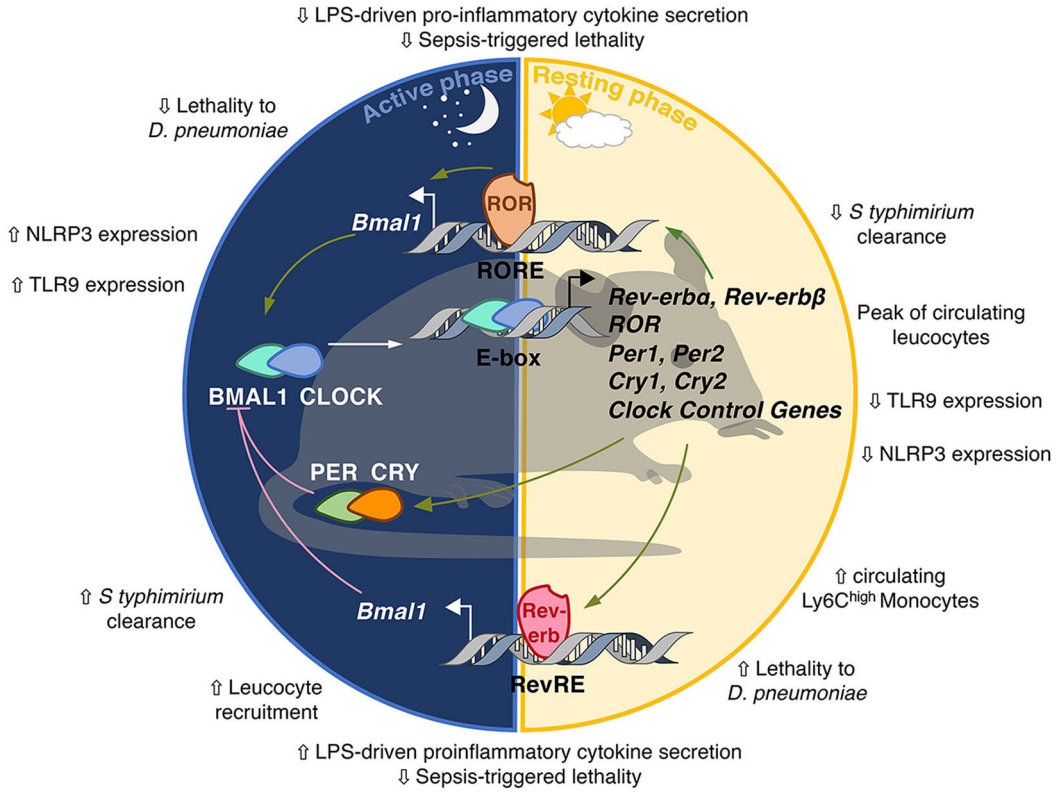

FIGURE 2 | The molecular circadian clock in mammals. The molecular clockwork is formed by transcription–translation feedback loops. The transcription factors BMAL1/CLOCK induce the expression of E-box-containing genes including the negative regulators Period (PER) and Cryptochrome (CRY). In turn, the PER/CRY heterodimer inhibits the transcriptional activity of BMAL1/CLOCK. Once PER and CRY levels are sufficiently low, a new cycle may start. CLOCK/BMAL1 induce the expression of the nuclear receptors Rev-erbα/β and retinoid-related orphan receptor α, β, and γ (RORα/β/γ). Rev-erbs and RORs interact with co-repressors (NCoR) and co-activators (NCoA) and compete for the binding of RevRE/RORE elements in common target genes to repress or activate, respectively, their transcription. Rev-erb and ROR are also able to repress E4BP4/Nfil3 which rhythmically inhibits D-box-dependent transcription. Additional layers of regulation of circadian gene expression include rhythmic histone modifications, circadian chromosomal 3D conformation and post-translational modifications such as acetylation, phosphorylation, sumoylation, O-GlcNacylation...The clock is involved in the control of so-called circadian immunity.

cells also results in K^+ and Ca^{2+} fluxes through P2X7 channel opening (23). In the same line, Ca^{2+} influx into the cytoplasm after mitochondrial reactive oxygen species (mtROS)-mediated cation channel transient receptor potential melastatin 2 (TRPM2) opening has been suggested to trigger the NLRP3 inflammasome assembly and IL-1β production in MSU-stimulated macrophages (28). The NLRP3 inflammasome is also sensing accumulation of aggregates (e.g., β-amyloid, Aβ) as well as metabolic stresses (8). Thus, the NLRP3 inflammasome is considered as a stress sensor that detects loss of homeostasis and abnormal endogenous molecules that signal infection, metabolic abnormalities or tissue damage (23).

CIRCADIAN CONTROL OF THE IMMUNE SYSTEM

Molecular Organization of the Mammalian Clock

The mammalian clock consists of transcriptional activators and repressors forming interlocked feedback regulatory loops and organized in positive and negative limbs that confer rhythmicity to each other (30) (**Figure 2**). The positive limb is driven by BMAL1 (Brain and Muscle ARNT-like 1) and CLOCK (Circadian Locomotor Output Cycles Kaput) which heterodimerize and bind to E-boxes in their target gene promoters, amongst which *Per* and *Cry* clock genes whose transcription is activated by BMAL1/CLOCK. Period (PER) 1/2/3 and Cryptochrome (CRY) 1/2 form the negative limb. PER and CRY, once they reach sufficient quantity in the cytoplasm, heterodimerize and translocate to the nucleus where they bind BMAL1-CLOCK heterodimers to inhibit BMAL1/CLOCK transcriptional activity in a rhythmic manner (30). This first circuitry is finely tuned by the nuclear receptors Rev-erbs and RORs (31), which compete for binding to the same RevRE/RORE and RevDR2 DNA sequences and regulate gene expression in an opposite manner. While Rev-erbs act as transcriptional repressors, RORs compete with Rev-erbs for DNA binding and activate transcription of common target genes, including *Bmal1* (32). Because Rev-erb isotypes display strong circadian rhythmicity in their abundance, this competition for binding to the *Bmal1* promoter is rhythmic and contributes to BMAL1 oscillations. It is noteworthy that these

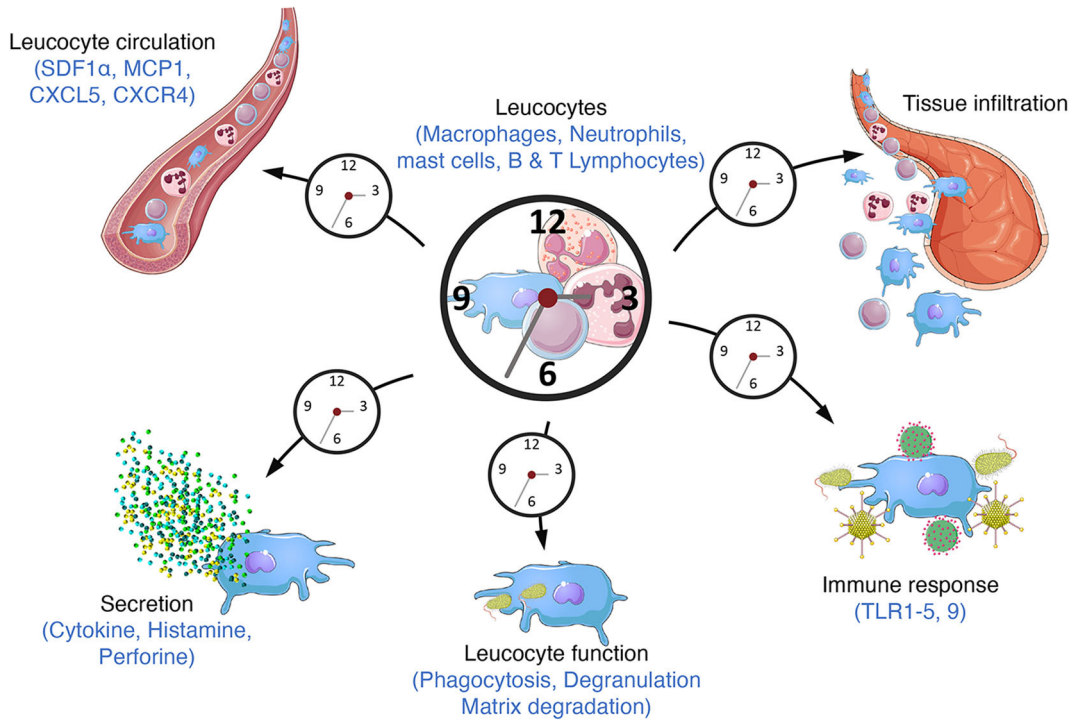

FIGURE 3 | Circadian immunity. The molecular clock regulates a large number of immune functions throughout the day/night cycle such as cytokine secretion, phagocytosis, response to pathogens (bacterium, parasite) through the expression of TLR9 or the TLR4 pathway and danger signals through the regulation of the NLRP3 inflammasome. Circulating leucocyte number also varies along the day altogether with their mobilization from bone marrow and their recruitment into tissues.

transcription factors not only control each other's transcription but also bind to numerous genes containing RORE/RevDR2 or E-boxes, thereby generating rhythmic transcriptional waves in transcriptional programs involved in local tissue functions. For instance, Rev-erb-α controls the expression of *E4bp4*/*Nfil3* in the liver (33, 34) but also in immune cells thereby regulating Th17 immune cell differentiation (35). Rev-erb also represses *Cry1* transcription thus controlling both limbs of the clock in a coordinated manner (36).

The rhythmicity observed in gene transcription is not only due to cyclic binding of these transcription factors but also to circadian variations in histone marks and chromatin organization at regulatory regions (30, 37). Beside epigenetic control, dynamic 3D chromatin architecture is another layer of circadian genome function (38). Additionally, post-translational modifications such as phosphorylation, SUMOylation, O-Glc-Nacylation are necessary to ensure the stability of these transcription factors and thus the pace and robustness of the clockwork [(39) for review].

Biological Clocks in the Immune System

Virtually all mammalian cell types harbor a functional circadian clock, leading to circadian oscillations in the transcriptome, proteome, and ultimately cell/tissue function. The central pacemaker is located in the suprachiasmatic nucleus of the hypothalamus. It receives light information and synchronizes clocks throughout the body according to this time cue. The clock is present in immune cells including macrophages, lymphocytes and neutrophils as well as in lymphoid tissues such as the spleen and lymph nodes (40). The number of circulating leukocytes oscillates diurnally, peaking during the rest phase, due to circadian variations in haematopoietic cell egress from bone marrow which preferentially occurs at the onset of this phase (**Figure 3**) (41, 42). In addition, tissue leucocytes display circadian variations mainly due to oscillations in their rolling and adhesion to the endothelium and infiltration into tissues which predominantly occurs at the onset of the active phase (43, 44). In parallel, immune cell functions such as cytokines production, phagocytosis of exogenous particles or response to pathogens also display daily oscillations resulting in time-of-day-dependent difference in the susceptibility to septic shock or injury (45–48). This temporal organization is meant to ensure an optimization of the immune response in order to maintain or rapidly and efficiently restore homeostasis after infection or injury/tissue damage. Consequently, clock disruption has often been associated with inflammatory diseases (**Figure 4**).

CIRCADIAN CONTROL OF THE NLRP3 INFLAMMASOME AND IMPLICATION IN PHYSIOLOGY AND PATHOLOGIES

Expression of the NLRP3 inflammasome complex components is low and increased transcription is achieved during the priming

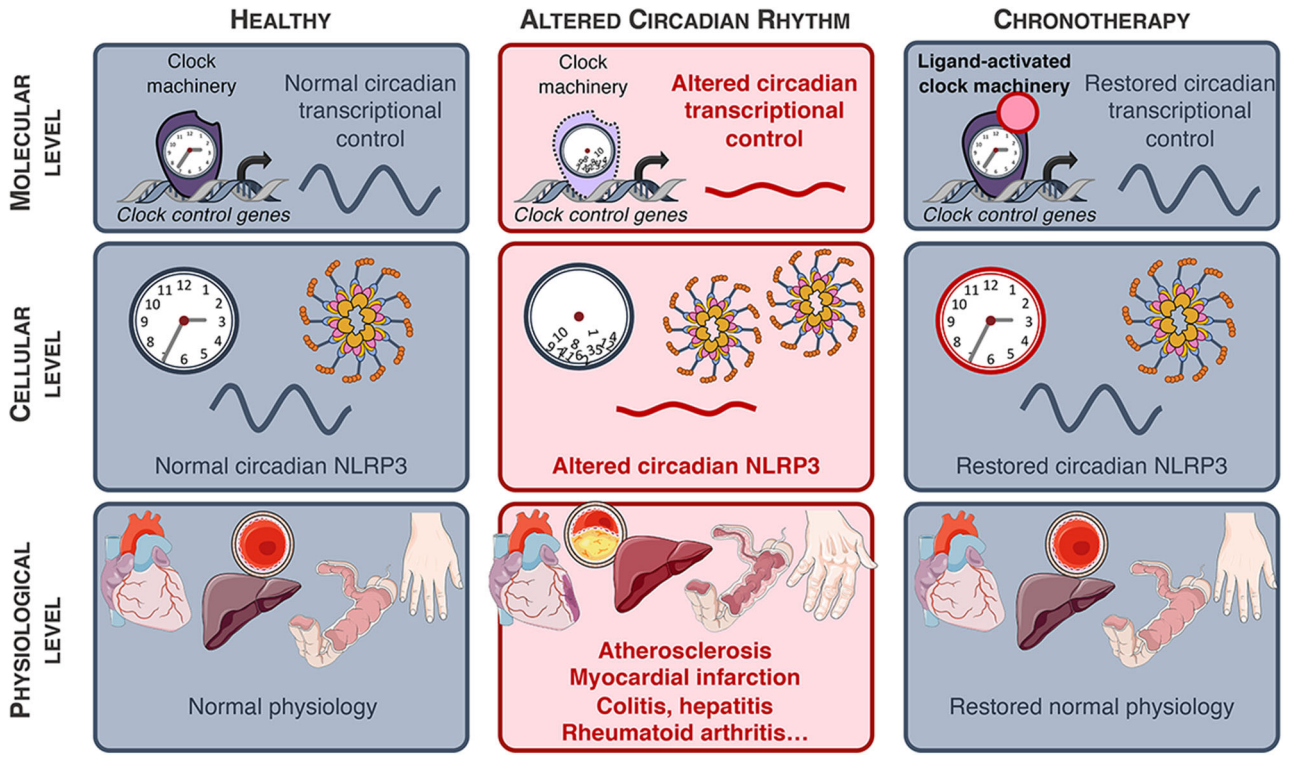

FIGURE 4 | NLRP3-associated diseases. NLRP3-associated diseases and potential for innovative chronotherapies.

step. It was recently demonstrated that the mRNA expression of the components of the NLRP3 inflammasome oscillates in a daily manner under the control of Rev-erbα in peritoneal mouse macrophages *in vivo,* reaching a peak (or zenith) during the active phase, corresponding to the lowest expression (or nadir) of Rev-erbα mRNA (21). Similar oscillations were observed in primary mouse bone marrow-derived and human monocyte-derived macrophages synchronized by a serum shock *ex vivo*, while circadian oscillations in *Nlrp3* mRNA were lost upon Rev-erbα ablation. NLRP3 protein amounts were accordingly regulated upon modulation of Rev-erbα activity (21). Strikingly, alteration in the NLRP3 pathway provoked by impairment of *Rev-erbα* expression triggers alterations in IL-1β and IL-18 secretion in peritoneum (21). Additionally, Rev-erbα also regulates the NLRP3 activation step. Indeed, *Rev-erbα* ablation led to increased speck formation, caspase-1 cleavage and NLRP3-induced caspase-1 mediated maturation and secretion of IL-1β and IL-18 in LPS-primed macrophages activated with nigericin or ATP. By contrast, activation of Rev-erb by its natural (heme) or pharmacological ligands reduced the secretion of these pro-inflammatory cytokines. Mechanistically, Rev-erbα binds to specific response elements in *Nlrp3* and *Il1β* gene promoters to silence their expression, and controls the NLRP3 inflammasome assembly and caspase 1 maturation (21) (**Figure 1**). In an *in vivo* model of acute sterile peritonitis induced by intraperitoneal administration of LPS and a concomitant injection of alum to specifically activate the NLRP3 pathway, IL-1β and IL-18 plasma levels were higher in *Rev-erbα*-deficient mice toward the end of the resting phase when Rev-erbα expression is highest in the wild-type controls, whereas the difference was lost during the active phase when Rev-erbα is nearly absent. Accordingly, and although this was not studied at different times of the day, Rev-erbα inhibition with an antagonist exacerbates the severity of LPS-induced acute lung injury by increasing NLRP3-dependent IL-1β secretion (49). These data demonstrate the rhythmic regulation of the NLRP3 inflammasome expression and activation, and suggest that Rev-erb pharmacological modulation may exert beneficial action in acute or chronic inflammatory diseases in which the NLRP3 inflammasome is over-activated, as detailed below.

Fulminant Hepatitis

Fulminant hepatitis (FH) is a life-threatening condition characterized by a fast-evolving hepatic dysfunction associated with encephalopathy and coagulopathy (50, 51). Numerous factors such as viral infection, metabolic and genetic diseases as well as absorption of toxic compounds are able to trigger FH, although overdose of acetaminophen still remains the main cause of FH nowadays (52). Acetaminophen accumulation leads to the production of large quantities of toxic metabolites provoking oxidative stress, mitochondrial membrane potential loss and hepatocellular death, the secretion of DAMPs and activation of the NLRP3 inflammasome (52). Increased IL-1β and activation of the NLRP3 inflammasome in macrophages has also been

TABLE 1 | NLRP3-associated diseases linked to the clock.

NLRP3-associated disease	Clock intervention with effects on NLRP3	References
Atherosclerosis	Not tested	
Colitis	• Rev-erbα KO, Bmal1 KO: worsening • Jetlag: worsening • Rev-erbα agonist: improvement	(56)
Fulminant hepatitis	• Rev-erbα KO: worsening • Rev-erbα agonist (mice): improvement • Chrono-pharmacological approach (mice)	(21)
Gout	Not tested	
Lung injury	Rev-erbα antagonist: worsening Rev-erbα agonist: improvement	(49)
Myocardial infarction/ischemia-reperfusion injury & heart failure	• Rev-erbα KO (mice, *in vivo*): worsening • Rev-erbα agonist (mice, *in vivo*, at ZT6/ZT18): improvement • Chrono-pharmacological approach	(57)
NASH	Not tested	
Rheumatoid arthritis	Not tested	
Type 2 Diabetes	Not tested	

shown in viral hepatitis (53–55). Strikingly, the susceptibility of FH is time-of-day dependent, upon the control of the molecular clock and Rev-erbα was identified as an important regulator of the inflammasome in this context. In mice, ablation of Rev-erbα led to exacerbated fulminant hepatitis, including increased liver damage that was blunted upon administration of the MCC950 specific NLRP3 inhibitor (**Table 1**) (21, 58). Remarkably, Rev-erbα pharmacological activation dramatically reduced liver injury thereby delaying death and improving the rate of survival from fulminant hepatitis from 10% in the control to 70% in the treated mice (**Figure 4**) (21).

Colitis

Several studies have suggested a role of the NLRP3 inflammasome in inflammatory intestinal diseases and although controversial results were first published, it is now accepted that NLRP3 activation is detrimental in this context. Ablating IL-18 or blocking its signaling reduced the severity of experimental colitis (59, 60). In addition, the NLRP3 inflammasome was identified as a central mediator of intestinal inflammation in dextran sulfate sodium (DSS)-induced colitis (61). Consistent with the previously described role of the clock in the regulation of the NLRP3 activation, DSS-induced colitis was found to be more severe in mouse models of environmental or genetic disruption of the clock (56). Confirming previous results, *Rev-erbα*-deficient mice were found to display increased activation of the NLRP3 pathway which accounted for the severe phenotype, whereas pharmacological Rev-erb activation attenuated colitis *in vivo* (**Figure 4**). Interestingly, when tested *in vitro,* the Rev-erb agonist seems to be active only on the priming step, and was ineffective at modifying caspase 1 maturation in cultured LPS-primed macrophages activated with ATP. This might be due to the fact that the cells were not synchronized in this study. Still, the effects of Rev-erb activation were abolished by MCC950, a specific inhibitor of the NLRP3 inflammasome activation.

Cardio-Vascular Diseases

Circadian Clock and Blood Vessels Physiology

Circadian clocks reside in the different cell types of blood vessels (62, 63) and participate in vascular function and tone (64). For instance, blood pressure displays circadian oscillations, starting to rise before the rest-to-active transition while being lower during sleep (65), coinciding with the higher frequency of acute cardiovascular events and the exacerbated acute thrombus formation in the early morning hours (66, 67). Circadian oscillations in clock genes expression are attenuated in human atherosclerotic plaque (68), suggesting a mechanistic link between altered clock function and vascular pathologies. Numerous studies revealed that clock disruption (e.g., altered sleep patterns, shift-work) increases cardiovascular risk factors such as dyslipidemia, diabetes, hypertension and lead to cardiovascular diseases including stroke and coronary heart disease (**Figure 4**) (66, 69). Several studies found a relationship between shift work or acute circadian misalignment and subclinical atherosclerosis, measured by higher intima-media thickness (IMT) and elevated systemic inflammation even after adjustment for age and common risk factors (70–73). Moreover, lower sleep duration and fragmented sleep are independently associated with an increased risk of subclinical coronary and non-coronary atherosclerosis (74).

Clock Control of NLRP3 Inflammasome Activation, IL-1β Production and Atherogenesis

Atherosclerosis is a lipid-driven inflammatory disease of the arterial wall. Infiltration and modification of lipoproteins in the subendothelial space result in their uptake mainly by macrophages, forming foam cells, thus initiating atherosclerotic lesion formation. Then, lipids (fatty acids, ox-LDL, cholesterol crystals...) accumulate as well as inflammatory cells, notably monocyte-derived macrophages, T and B lymphocytes (75–77). Inefficient efferocytotic removal of these foam cells and apoptotic cells promote lesion progression toward advanced lesions with a necrotic core, degradation of the extracellular matrix, migration of smooth muscle cells and in some cases calcification, which may become vulnerable (78). Genetic alteration of the molecular clock contributes to metabolic imbalance and inflammation which promote atherogenesis (79, 80). For instance, BMAL1 modulates lipoprotein production and biliary cholesterol excretion, and its ablation led to hyperlipidemia and atherosclerosis (81). In the same line, Rev-erbα diminishes atherogenic lipoproteins plasma levels (82), modulates the inflammatory profile of macrophages toward an anti-inflammatory phenotype (83) while its activation reduced atherogenesis (84). Accordingly, BMAL1 regulates macrophage polarization as well as the cyclic trafficking of Ly6C^{hi} monocytes and myeloid *Bmal1* deletion increased monocyte recruitment and worsened atherosclerosis (**Figures 2**, **3**) (85). Pro-inflammatory recruitment through the CCL2 (MCP-1)-CCR2 axis plays an important role in plaque development (86). In a recent study, McAlpine and colleagues revealed that sleep modulates haematopoiesis while chronic sleep

fragmentation in a mouse model prone to atherosclerosis resulted in increased production of Ly6C^{high} monocytes and aggravated atherosclerosis development due to increased infiltration to the lesions (**Figure 4**) (87). In line, disruption of circadian rhythms by chronic jetlag obtained by weekly alternating light-dark cycles with 12 h shifts enhanced atherosclerosis development and increased lesion macrophage content (**Figure 4**) (88). Interestingly, Winter et al. elegantly showed that myeloid cells are recruited to the lesions in a circadian manner, with a peak during the active-to-rest transition, through the rhythmic deposit of CCL2 on the arterial endothelium by circulating cells. A chrono-pharmacological approach targeting monocyte recruitment *via* timed inhibition of the CCR2/CCL2 axis during the active phase dampened atherosclerotic lesions development (**Figure 4**) (89).

In atherosclerotic lesions, oxLDL can prime the macrophage NLRP3 inflammasome by activating TLRs-dependent pathways. In addition, CD36-mediated oxLDL uptake eventually results into intra-lysosomal crystallization. Together with phagocytized extracellular cholesterol, they are thought to trigger macrophage lysosomal damage thus provoking cathepsins release (18). Moreover, defective cholesterol efflux in myeloid cells results in accumulation of unesterified cholesterol which contributes to both priming and activation of the NLRP3 inflammasome, promoting neutrophil recruitment and neutrophil extracellular trap (NET) formation in atherosclerotic plaques (90). The NLRP3 inflammasome activation contributes to the vascular inflammatory response through enhanced production of IL-1α and IL-1β, the latter driving inflammation during early atherogenesis and the evolution of advanced atheroma in mice (91). Canakinumab is an IL-1β-neutralizing antibody approved for the treatment for CAPS-associated symptoms which also reduced the incidence of two other NLRP3-related diseases, arthritis and gout (92). Recently, the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) study demonstrated that IL-1β neutralization decreased the incidence of atherosclerotic disease and reduced systemic inflammation in at-risk patients with previous myocardial infarction in the absence of effect on lipids, indicating that suppressing IL-1β contributes to the reduction in cardiovascular risk (93). However, substantial residual inflammatory risk still subsisted after IL-1β neutralization, with on-treatment IL-18 and IL-6 plasma levels associated with future cardiovascular risk (94), advocating for therapies that simultaneously inhibit IL-1β and IL-18. In mice, inhibition of the NLRP3 inflammasome reduces atherogenesis in *ApoE*$^{-/-}$ or *LDLr*$^{-/-}$mice (24, 95). Although plausible, it is currently unclear whether circadian control of NLRP3 inflammasome activation is perturbed within macrophage foam cells from atherosclerotic lesions. In this perspective, a therapy that targets the clock, and particularly Rev-erbα, in a chrono-pharmacological approach would be worth testing as Rev-erbα not only regulates NLRP3 inflammasome expression and activation, reducing both IL-1β and IL-18, but also MCP-1 expression and IL-6 production by macrophages (**Figure 4**) (47, 96), as well as lipoprotein metabolism, thereby simultaneously impacting both local inflammation and systemic risk factors.

Myocardial Infarction and the Circadian Control of NLRP3 Expression and Activation

The NLRP3 Inflammasome Is Activated Upon Acute Myocardial Ischemia/Reperfusion Injury

Myocardial infarction (MI) is one of the leading causes of death worldwide and is associated with a poor quality of life, acknowledging the increased interest in finding novel therapeutics to reduce reperfusion injury and preserve cardiac function. Despite improvement in reperfusion and treatment strategies that have led to higher survival rates, fibrosis and adverse left ventricular remodeling consecutive to reperfusion injury leads to cardiac contractile dysfunction and eventually heart failure (97).

Acute myocardial infarction (AMI) initiates a sterile inflammatory response that enables necrotic cardiomyocyte debris removal, angiogenesis and wound healing (98); however, this inflammatory response also promotes cell death by pyroptosis, expanding infarct size, and results in fibrosis and adverse ventricular remodeling. Then, refined intervention to rapidly attenuate this inflammatory burst is desirable (99). IL-1β and IL-18 are rapidly increased upon MI. Interestingly, administration of IL-1β- or IL-18-neutralizing antibody inhibits cardiomyocyte apoptosis, reduces infarct size and improves cardiac dysfunction after MI in mice (100, 101). In line, reduction of IL-1β production in caspase1- and in ASC-deficient mice upon ischemia/reperfusion is associated with a marked reduction in the infarct size, left ventricle remodeling and myocardial fibrosis (102). These data suggested that activation of the inflammasome may provoke further tissue damage through caspase-1-mediated production and release of IL-1β. Sandanger et al. confirmed that *Nlrp3* deletion in mice leads to reduced infarct size and preservation of cardiac function in isolated perfused hearts subjected to acute I/R *ex vivo* (103).

Expression of the NLRP3 inflammasome components is very low and priming is induced during ischemic injury by cellular debris. NLRP3 is then activated by extracellular ATP as well as cardiolipin and mtDNA released by dying cells from damaged tissue after acute ischemic injury, or within minutes of reperfusion due to sudden surge of reoxygenation-induced ROS production and mitochondrial damage (**Figure 1**) (98). The NLRP3 inflammasome also senses extracellular-mediated efflux of K^+ in cardiac fibroblasts upon hypoxia (103). In addition, the NLRP3 inflammasome is activated by numerous danger signals stemming from co-morbidities such as high glucose and lipid levels and derivatives (ceramides, advanced glycation products, which may lead to chronic activation of the NLRP3 inflammasome locally or in other organs) (104).

Inhibition of the NLRP3 Activation as a Novel Strategy to Reduce Myocardial I/R Injury?

Strategies to inhibit the activation of NLRP3 in the early reperfusion period after ischemic MI to reduce infarct size, avoid adverse remodeling and fibrosis and ameliorate cardiac function have been tested. Several inhibitors of the NLRP3 inflammasome

activation have been developed, enabling pharmacological intervention in animal models undergoing AMI. Administration of the NLRP3 inhibitor MCC950 (58) lowers infarct size and area at risk (105, 106). Remarkably, NLRP3 inhibition by MCC950 treatment was associated with preserved left ventricle (LV) ejection fraction (LVEF), reduced fibrosis and myocardial immune cell infiltration. Interestingly however, the benefit of administering the NLRP3 inhibitor before AMI or within 1 h of reperfusion was lost when the NLRP3 inhibitor was given after 3 h of reperfusion, suggesting that inhibition should be achieved at time of NLRP3 assembly and activation (107). Other NLRP3 inhibitors have been shown to reduce infarct size in mouse models of myocardial ischemia/reperfusion. Among them, OLT1177 reduces infarct size in mice (108) and is currently in Phase 1b in a randomized, double-blinded, placebo-controlled, safety, and pharmacodynamics study in 30 subjects with stable systolic heart failure (HF) with impaired LVEF.

Circadian Rhythms in Cardiac Biology and Diseases

Daily oscillations of blood pressure and heart rate are reduced or lost in cardiomyocyte-specific Clock-mutant (109) acknowledging the important role of cardiomyocyte clock machinery in cardiac function. Consistently, circadian disruption due to either environmental out-of-sync stimuli or genetic manipulation of clock genes results in cardiomyopathies, cardiac dysfunction, arrhythmia, and reduced survival (110–113) and for review (114) (**Figure 4**). Additionally, circadian variations are seen in the onset and frequency of myocardial infarction, stroke and sudden death (115, 116), as well as in the severity of the diseases (117). Furthermore, environmental circadian disruption adversely impacts cardiac remodeling and function, increases macrophage infiltration and led to cardiac hypertrophy in mice undergoing MI (**Figure 4**) (118). The circadian influence in the tolerance to I/R injury was corroborated in mice undergoing I/R at the resting-to-active and active-to-rest transitions. The former led to exacerbated infarct size, and subsequent fibrosis and adverse cardiac remodeling. This time-of-day difference in the tolerance to I/R was markedly attenuated in cardiomyocyte-specific circadian clock mutant mice (119). In humans, it was recently assessed whether myocardial tolerance of I/R differed depending on the timing of aortic valve replacement surgery, as measured by the occurrence of major adverse cardiovascular events (cardiovascular death, myocardial infarction, and admission to hospital for acute heart failure). Expectedly, perioperative myocardial injury was better tolerated when patients underwent surgery in the afternoon (120). Interestingly, targeting the circadian clock through pharmacological modulation of Rev-erbα/β in mice was able to reduce myocardial I/R injury *ex vivo* in an isolated Langendorff-perfused mouse heart model of hypoxia-reperfusion, thus providing new therapeutic ways to dampen the adverse outcome of cardiac I/R injury. Whether Rev-erbα might be an interesting target to reduce cardiac dysfunction was further established in a model of transaortic constriction-induced heart failure (121) as well as in a mouse model of AMI (122). In this later report, the author suggested that blunted inflammation and reduced recruitment of neutrophils and pro-inflammatory macrophages upon pharmacological Rev-erbα modulation may, at least in part, contribute to the benefit of targeting Rev-erbα. Remarkably, Schloss et al. elegantly demonstrated that infarct size is higher at ZT13 vs. ZT5 when the number of cardiac Ly6C^{high} monocytes is highest likely because of increased CCR2-mediated recruitment of these cells, and that blocking the CCR2-CCL2 axis blunted the time-of-day variations in infarct size (123). These data, together with the observation that Rev-erbα controls macrophage NLRP3 activation (21), point to a possible role of monocyte/macrophage Rev-erbα in I/R tolerance. In a recent report, Martino and colleagues questioned the cell-specific role of Rev-erbα in the protective effect of Rev-erbα activation. They found that activating Rev-erbα at time of reperfusion in wild-type mice limits infarct expansion, improves cardiac function and outcomes, and reduced recruitment of neutrophils and macrophages as well as cardiac NLRP3 inflammasome activation. However, myeloid cells were unlikely to account for this beneficial effect as shown by bone marrow transfer experiments (57). Instead, Rev-erbα may downregulate the NLRP3 inflammasome in cardiac fibroblasts although further studies using cell-specific mutant mice are necessary to pinpoint the exact contribution of each cell types. More importantly, pharmacological Rev-erbα activation showed the greatest benefit when given at time of reperfusion, whenever it happened during the active (ZT18) or resting (ZT6) phase, although the benefit was greater at ZT6 corresponding to maximal Rev-erbα expression (**Figure 4**). This suggests that beyond the time of treatment and potentially differential effect on the pace of the clock, Rev-erbα-regulated inhibition of NLRP3 before or at reperfusion may hold promise to reduce myocardial I/R damages, whenever the time at which the patient will undergo cardiac surgery or experience MI.

CONCLUSION

In this review, we have highlighted the relationship between circadian immunity and the NLRP3 inflammasome pathway. As a central sensor of tissue damages and metabolic imbalance, NLRP3 plays a pivotal role in tissue homeostasis in many tissues including liver, heart and the vasculature. Sustained activation of NLRP3 by exogenous or endogenous triggers thus aggravates chronic inflammatory diseases such as atherosclerosis (24) or worsen acute inflammatory conditions such as fulminant hepatitis (21) or myocardial infarction (121). As such, the NLRP3 inflammasome represents an innovative target, as exemplified by the use of NLRP3 inhibitors in several disease models. However, MCC950 displays hepatotoxic properties, advocating for the development of alternative NLRP3 inhibitory strategy (124). Strikingly, because NLRP3 is controlled by the clock machinery, the time of exposure to intruders and their sensing has a dramatic impact on the inflammatory response amplitude, the disease outcome and its resolution. As such, a chrono-pharmacological approach targeting NLRP3 may have greater benefits for the treatment of NLRP3-driven diseases (**Figure 4**). Since pathological tissues often

display distinct circadian oscillation patterns compared to healthy tissue, such strategies would allow to target NLRP3 specifically in pathological areas and then preserve homeostasis in healthy tissue and thus reduce adverse effects. Several targets should be considered, either the NLRP3 pathway itself or NLRP3-regulating clock components such as Rev-erbα. Finally, alteration of NLRP3 pathway is involved in other diseases including diabetes, Alzheimer disease, gout, rheumatoid arthritis, or asthma (5). Clock-driven NLRP3 resynchronisation may represent an additional approach to help treating these diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Some of our work included in this review manuscript was supported by INSERM, the ANR-Labex-EGID (EGID, ANR-10-LABX-46), the CPER-CTRL Institut Pasteur de Lille, the Fondation Francophone pour la recherche sur le diabète (FFRD) together with the Fédération Française des Diabétiques (AFD) AstraZeneca, Eli Lilly, Merck Sharp & Dohme (MSD), Novo Nordisk & Sanofi; the Région Hauts-de-France/FEDER (Chronoregeneration), Association Française contre les Myopathies (AFM), Société Francophone du Diabète (SFD), Fondation de France and the ANR.

ACKNOWLEDGMENTS

We would like to thank the members of the HD's team for helpful discussions.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 04 August 2020
doi: 10.3389/fimmu.2020.01743

Innate Rhythms: Clocks at the Center of Monocyte and Macrophage Function

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The circadian cycle allows organisms to track external time of day and predict/respond to changes in the external environment. In higher order organisms, circadian rhythmicity is a central feature of innate and adaptive immunity. We focus on the role of the molecular clock and circadian rhythmicity specifically in monocytes and macrophages of the innate immune system. These cells display rhythmicity in their internal functions, such as metabolism and inflammatory mediator production as well as their external functions in pathogen sensing, phagocytosis, and migration. These inflammatory mediators are of clinical interest as many are therapeutic targets in inflammatory disease such as cardiovascular disease, diabetes, and rheumatoid arthritis. Moreover, circadian rhythm disruption is closely linked with increased prevalence of these conditions. Therefore, understanding the mechanisms by which circadian disruption affects monocyte/macrophage function will provide insights into novel therapeutic opportunities for these chronic inflammatory diseases.

Keywords: circadian, macrophage, monocyte, molecular clock, inflammation, cell migration, immunometabolism, phagocytosis

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Bert Maier, Charité—University Medicine Berlin, Germany
Adam C. Silver, University of Hartford, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *24 April 2020*
Accepted: *30 June 2020*
Published: *04 August 2020*

Citation:
Timmons GA, O'Siorain JR, Kennedy OD, Curtis AM and Early JO (2020) Innate Rhythms: Clocks at the Center of Monocyte and Macrophage Function. Front. Immunol. 11:1743. doi: 10.3389/fimmu.2020.01743

INTRODUCTION

Circadian rhythms are oscillations in physiology and behavior with a 24-h periodicity. This rhythmicity first arose at the cellular level, ∼2.5 billion years ago. Organisms evolved this strategy as an adaptation to rhythmic changes in oxidative stress caused by the rotation of the earth on its axis (1). A common hypothesis is that rhythmic cycles of peroxiredoxins conferred a selective advantage on photosynthetic bacteria, allowing them to detoxify reactive oxygen species (ROS) derived from daily oxygen consumption. Today, mammalian circadian rhythms are more complex and molecular clocks throughout the body can synchronize physiological and behavioral activities to appropriate times of the 24-h day, thus maximizing energy efficiency (2–4).

The term "circadian" was coined by Franz Halberg in 1959. It was Halberg who carried out a seminal study showing that survival rates in mice were dependent on the time-of-day when *Escherichia coli (E. Coli)* endotoxin was injected (5). Interestingly, the response to endotoxin relies heavily on cells of the innate immune system, the branch of immunity which provides the first line of defense against infection and damage. Monocytes and macrophages are central to innate immunity (6) and their molecular clocks have been implicated in multiple inflammatory disorders (7). Monocytes are short-lived, motile cells found in blood, bone marrow, and spleen

(6). They quickly respond and migrate to sites of infection. They are often considered a systemic reservoir of myeloid precursors, important in the renewal of tissue macrophages and dendritic cells. Macrophages, on the other hand, are long-lived tissue-specific cells with roles ranging from tissue homeostasis to immune response generation against pathogens (6). In this review, we will discuss our understanding of the molecular mechanisms governing circadian control of monocytes/macrophages and their potential impact on chronic inflammatory disease.

THE MOLECULAR CLOCK

Virtually all cell types have an internal molecular clock (8, 9). However, the master-clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN processes external light signals, generating rhythmic signals via the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system, which synchronize peripheral clocks in tissues (10, 11). The molecular clock is regulated by a series of interlocking transcription-translation feedback loops (TTFLs), powered by the heterodimeric pairing of BMAL1 and CLOCK (**Figure 1A**). BMAL1 is the master clock regulator and its deletion ablates most rhythmic activity (12). BMAL1:CLOCK heterodimers bind E-box sites on DNA and facilitate the transcription of clock-controlled genes (CCG). Included in CCGs are the clock's negative regulators, period (PER) and cryptochrome (CRY), which translocate to the nucleus, disrupt the BMAL1:CLOCK heterodimer and inhibit their own expression (13–15). Other regulators within this circuitry include RAR-related orphan receptor alpha (RORα) and the nuclear receptor REV-ERBα, which promote and suppress *Bmal1* transcription, respectively (16, 17). Comprehensive details of the molecular circadian circuitry have been reviewed elsewhere (18, 19).

A powerful example of the clocks influence is that 43% of murine protein-coding genes across 12 organs display circadian cycling, in an organ-dependent manner (8). In baboons, a closer cousin of humans, an astonishing 80% of protein-coding genes across 64 tissues displayed rhythmicity (9). Monocytes and macrophages also express a robust molecular clock (20–22) and at least 8% of transcripts in murine peritoneal macrophages are circadian (23). Many of these cycling transcripts are involved in key innate-immune functions, such as antigen presentation, immune regulation, and phagocytosis (23). Given that their primary role is to sense and respond to challenges from pathogens, which would be driven by rhythms in feeding and activity, it is unsurprising that significant rhythmicity has been documented in monocyte and macrophage function (**Figure 1B**).

PATTERN RECOGNITION RECEPTORS

Monocytes and macrophages use membrane-bound pattern recognition receptors (PRRs), to sense the external environment for infection and damage. PRRs are capable of recognizing pathogen-associated or damage-associated molecular patterns (PAMPs/DAMPs) facilitating responses to harmful substances (24). PAMPs include non-self-molecules, such as bacterial deoxyribonucleic acids and lipoproteins, which represent infection (25). DAMPs are endogenous molecules that represent deviation from homeostasis to the body, e.g., adenosine triphosphate (ATP) and DNA, which are released from cells with cell-death or damage. Perhaps the most well-studied group of PRRs are the toll-like receptors (TLRs), which induce a series of signaling cascades that convert monocytes and macrophages from quiescence to immunologically active (26).

TLR4, a surface bound receptor, senses the endotoxin LPS found on gram-negative bacteria such as *E. coli* and *Salmonella* (27). Halberg's observation that *E. coli* endotoxin-induced death varied with time-of-day of injection suggested that TLR4 receptor signaling was under circadian control (5). A more recent study found that mice injected with LPS at Zeitgeber time (ZT) 0 (lights on and beginning of rest phase) were less likely to succumb to disease than mice injected at ZT12 [(lights off and beginning of active phase (28)] (**Figure 1B**). Deletion of *Bmal1* in myeloid cells, which includes monocytes and macrophages, resulted in loss of time-of-day protection. However, circadian oscillation in *Tlr4* expression has not been observed in macrophages (29), but many genes downstream of TLR4 were cycling (23) such as *IkBα*, which negatively regulates NF-κB, and *Adam17*, a metalloproteinase involved in TNFα release (30). Direct circadian impact on other TLRs, such as TLR9, an intracellular receptor that senses bacterial and viral DNA, has been observed (29). In a TLR9-dependent model of sepsis, greater lethality was observed at the time-of-day coinciding with highest TLR9 expression in splenic macrophages and B cells. In splenic macrophages, rhythms have been also observed in the mRNA expression of *Tlr2 and Tlr6,* peaking at ZT14 (31). Therefore, circadian control in some TLRs and downstream signaling pathways appears as an important mechanism directing circadian inflammation. However, circadian TLR expression differs amongst immune cells (31), and an explanation of this, and the functional consequences of it, are still largely unknown.

IMMUNOMETABOLISM

Immune cell activation following PRR stimulation requires significant amounts of energy. Immunometabolism is an emerging field seeking to understand how cellular metabolism impacts immunity (32, 33). Multiple relationships exist between clock function and metabolism in liver (34–37) and muscle (38–40). However, the specific relationships between the molecular clock, metabolism, and immunity have yet to be fully determined (41, 42). Many immunometabolism investigations have been on macrophages, whose activity covers a spectrum of phenotypes. LPS stimulation promotes a pro-inflammatory M1-like phenotype characterized by increased glycolysis and decreased oxidative phosphorylation (43). In contrast, M2-like stimuli, such as IL-4, decrease glycolysis, promote oxidative phosphorylation, and generating an anti-inflammatory state (44). These metabolic shifts can directly impact outcomes of certain pathologies, such as sepsis (45). Interestingly, BMAL1 suppresses sepsis through its impact upon glycolytic metabolism

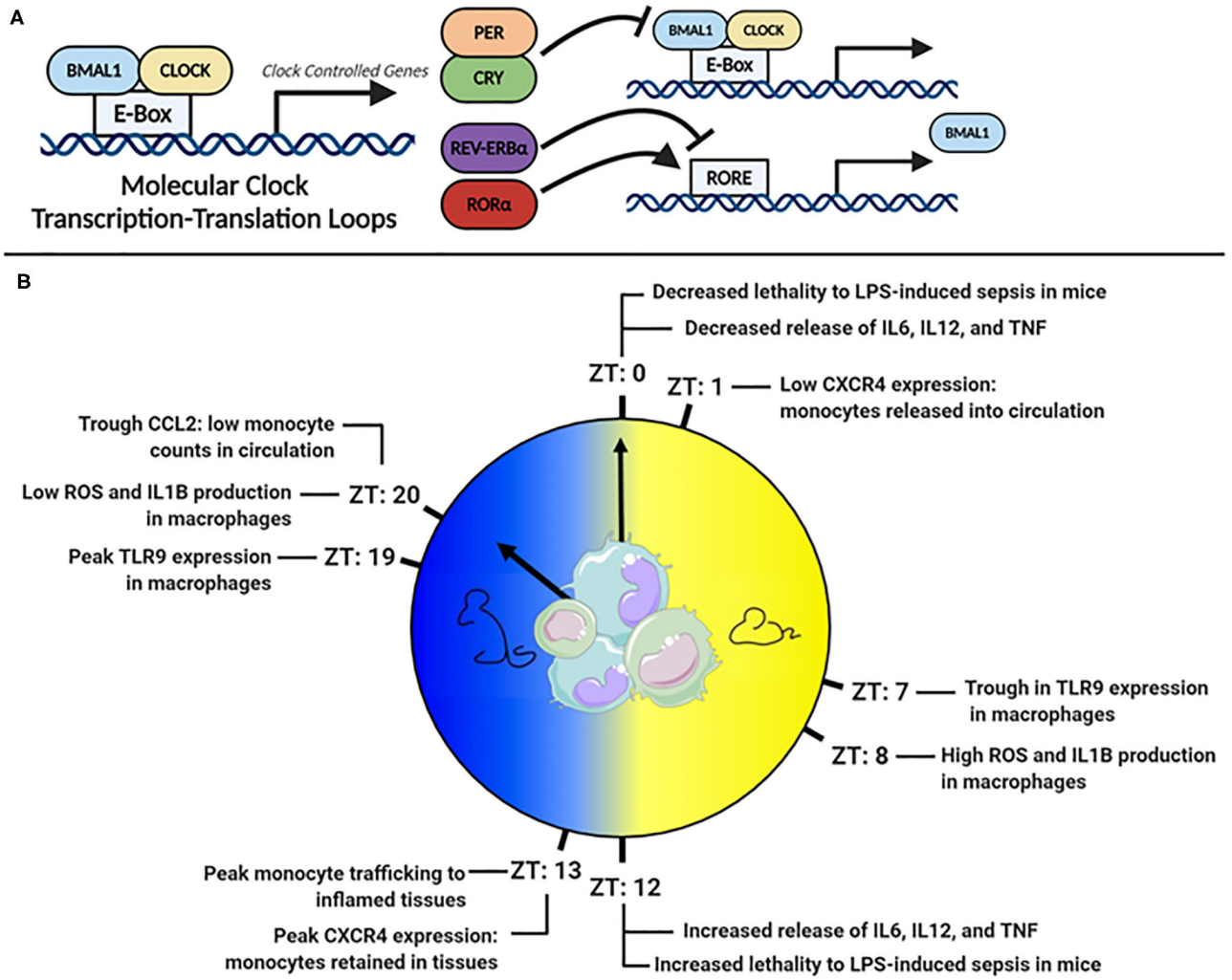

FIGURE 1 | (A) The molecular clock transcription factor feedback loop. **(B)** Peaks troughs in murine monocyte and macrophage inflammatory mediators across the 24 h day.

(46). BMAL1 transcriptionally targets the glycolytic enzyme *Pkm2*, negatively regulating glycolysis, lactate production, and the immune checkpoint protein PD-L1. Deletion of *Bmal1* in macrophages diminished their ability to control glycolysis and increased downstream PD-L1/T-cell mediated septic shock, revealing the importance of macrophage clock function on metabolic control of inflammation.

Mitochondria have extended their reach beyond energy production and are now considered central hubs of immunity (47, 48). They achieve this through production of metabolites (47) and ROS (49), and by altering their morphology, which can affect metabolism and signal transduction pathways (48). Mitochondrial morphology describes the elongation (fusion) or segmentation (fission) of mitochondria within the cell (50–53). Rhythms in mitochondrial morphology and membrane potential have been observed in synchronized peritoneal macrophages *in vitro* (54). However, whether mitochondrial morphology is under circadian control in innate immunity is still unknown. Thus, determining circadian immunometabolism of the innate immune system will provide new insights into a range of diseases and pathologies.

Inflammatory Mediators

While classifying macrophages into M1 vs. M2 is convenient, the reality is that macrophages *in vivo* are highly plastic cells existing across a spectrum of activation states (55, 56). Nonetheless, macrophages isolated from mice lacking the clock genes *Per1 and Per2* preferentially display an M1-like pro-inflammatory phenotype. However, this phenotype is attenuated following overexpression of PPARγ (57), a critical regulator of M2-like macrophage polarization (58). The circadian hormone melatonin promotes an M2-like phenotype, acting through the clock component RORα and through metabolite-signaling dependent mechanisms (59, 60). When mice were exposed to

a shifted light-dark cycle and fed a high-fat diet, more M1-like macrophage polarization was observed compared to normal light-dark cycles controls (61). IL-6 expression in peritoneal macrophages isolated from chronically clock-disrupted mice was increased following exposure to LPS (62). The response of individual macrophages to LPS has been shown to be dependent on the circadian genes *Nfil3* and *Dbp* (63). These two genes are in antiphase to each other and have opposite effects on LPS induced inflammation. NFIL3 and DBP competitively bind to the promoter of *Il12b* repressing and enhancing its expression, respectively. The oscillations of these circadian proteins provide variation in the response to LPS across the circadian day. This study highlights the molecular clock as a potential mechanism by which genetically identical cells of the same lineage may respond differently to the same stimuli. Taken together, data from these studies illustrate the importance of circadian rhythmicity and the molecular clock on macrophage polarization.

Our current understanding of clock control of macrophage/monocyte expression of chemokines and cytokines is summarized in **Figures 2A,B**, respectively. Serum levels of IL-6, IL-12, CCL5, CXCL1, and CCL2 were shown to be higher in WT mice injected with LPS at the time of transition to dark phase. Similarly, serum levels of CCL2, IL-1β, IL-6, and IFN-γ were higher in mice infected with *Listeria* at ZT8 vs. ZT0 (22) and deletion of *Bmal1* in the myeloid lineage of mice also increased these cytokines (22). BMAL1 directly induces the master antioxidant transcription factor NRF2, which diurnally regulates ROS in myeloid cells, limiting HIF-1α induced IL-1β (64). Others have demonstrated increased *Hif1a* and *Il1b* expression in macrophages with deletion of *Bmal1* (65). This was due to a loss of BMAL1's epigenetic role in down regulating TLR4 responsive enhancer RNA (eRNA) expression (65). CLOCK enhances gene expression of *Il-6*, *Il1b*, *Tnfa*, *Cxcl1*, *Ifnb*, and *Ccl2* (66). CLOCK also boosts NF-kB activity in mouse endothelial fibroblasts (MEFs), while BMAL1 works to sequester CLOCK from the NF-κB subunit p65 in MEFs (67). Whether this mechanism exists in macrophages is unknown but warrants investigation. However, *Bmal1* deletion in myeloid cells is known to increase expression of p65, mediated by miR-155, with a subsequent increase in TNFα. TLR4 activation by LPS also results in increased levels of miR-155, which targets *Bmal1* for degradation, potentiating inflammation (28). This evidence suggests that BMAL1 suppresses, while CLOCK potentiates, the inflammatory response in macrophages. Further work is needed to clarify the direct and indirect regulatory mechanisms impacted by these circadian transcription factors.

REV-ERBα also has a role in regulating inflammation in macrophages. Global *Rev-Erbα* deletion ablates time-of-day gating of peritoneal macrophage IL-6 production. Alveolar macrophages isolated from these mice have heightened inflammation in terms of *Il6*, *Ccl2*, and *Ccl5 expression* (68). Conversely, a REV-ERBα agonist suppresses expression of *Il6*, *Il19*, *Cxcl6*, *Cxcl11*, and *Ccl2* in LPS-stimulated human monocyte-derived macrophages (69). REV-ERBα directly binds to the promoter of Ccl2 (70) attenuating its expression. However, RORα binding promotes *Ccl2* expression (70). REV-ERBα has additional anti-inflammatory function via recruitment of the NCoR-HDAC corepressor complex, inhibiting eRNA transcription, and subsequent downstream mRNA transcription of the inflammatory genes *Cx3cr1* and *Mmp9* (71). In terms of neuroinflammation, REV-ERBα negatively regulates microglial expression of *Il1b*, *Il6*, and Ccl2 (72). In a murine model of DSS-induced colitis NF-κB signaling is also increased with *Rev-Erbα* deletion (72, 73), and interestingly this was shown to promote indirect activation of the NLRP3 inflammasome. A model of fulminant hepatitis (74) demonstrated direct negative regulation of *Nlrp3* mRNA by REV-ERBα (74). Thus, a wealth of evidence is emerging that REV-ERBα through various mechanisms, is a suppressor of inflammation in macrophages.

Per1/Per2 also impacts macrophage inflammatory responses. Mice lacking these genes have increased expression of *Il1b* and *Tnfa* basally, as well as in response to LPS (57). The repressive effects of the PER complex are mediated through PPARγ. PER1 and PPARγ bind the *Ccr2* promoter to inhibit its expression. Deletion of *Per1* increases expression of *Ccr2* and migratory activity in macrophages (75). Peritoneal macrophages lacking *Per2* have heightened responses to TLR9 activation displaying heightened TNFα and IL-12 production (29). CRY also suppresses the inflammatory response in macrophages via negative regulation of the cAMP-PKA-NF-κB pathway (76). Loss of CRY results in constitutive upregulation of *Il6*, *Tnfa*, and *inos*. Thus, the PER/CRY complex is another mechanism of clock-related suppression of inflammatory mediators in macrophages.

Thus, the activation state and regulation of cytokines, chemokines, ROS, miRNAs, and eRNAs in monocytes and macrophages are directly and indirectly targeted by components of the circadian machinery. Circadian regulation of these mediators ensures a closely controlled and appropriately timed macrophage/monocyte response to challenge and infection.

PHAGOCYTOSIS

A crucial function of macrophages is the ingestion of pathogens via phagocytosis. Diurnal regulation of phagocytosis has been demonstrated in *ex vivo* peritoneal macrophages (20). Synchronized peritoneal macrophages *in vitro*, suggested circadian rhythmicity in phagocytic activity (54). Another study showed similar time-of-day variation in peritoneal macrophage phagocytosis *ex vivo,* but found that this pattern was lost *in vivo* (77). A recent study demonstrated deletion of *Bmal1* creates a more phagocytic, motile, and ultimately antimicrobial macrophage via a RhoA-dependent mechanism, which impacted on *Streptococcus pneumoniae* lung infection. This is an extremely interesting development, however, whether BMAL1 and other clock components play this role in response to the full range of pathogens and foreign bodies that macrophages can phagocytose, is yet unknown.

CIRCADIAN MIGRATION

Appropriate cell migration of immune cells into tissues is critical for protective immunity. Monocyte migration is highly rhythmic

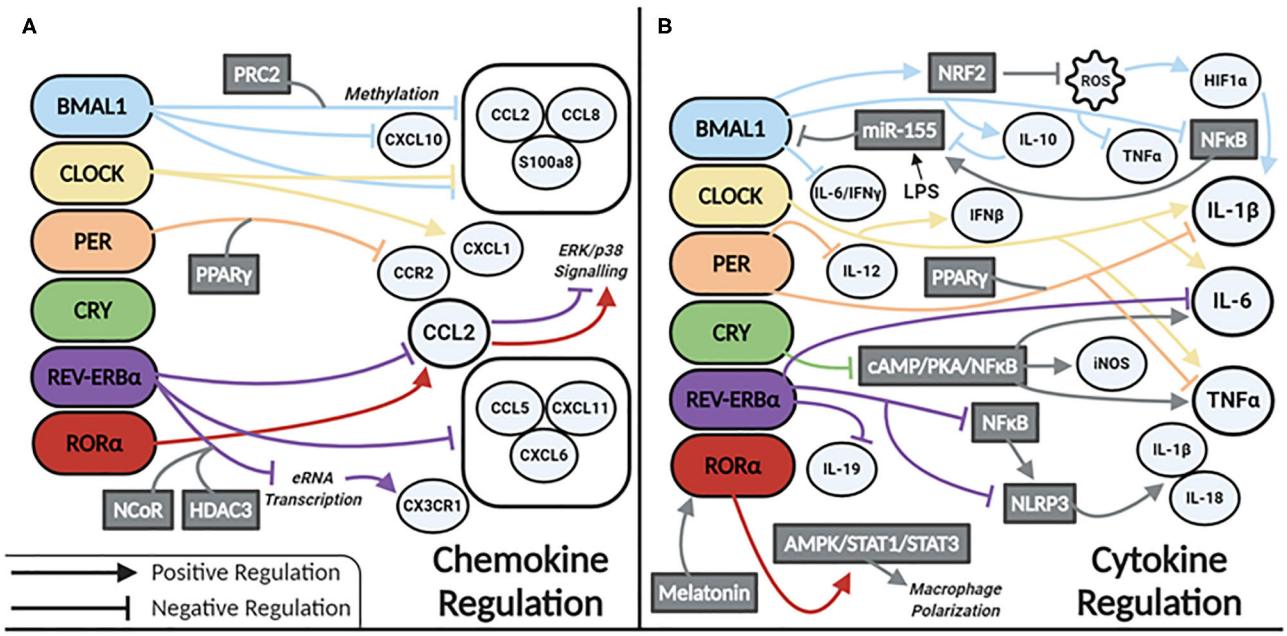

FIGURE 2 | Circadian clock proteins mediated molecular regulation of **(A)** chemokines and **(B)** cytokines.

and is tightly controlled by autocrine monocyte signaling as well as from the destination tissue. In mice, total-blood leukocyte numbers peak during the behavioral rest phase (ZT5), whereas their migration into bone-marrow and organ tissues peaks during the behavioral active phase (ZT13) (78). Central to regulating monocyte trafficking into tissues is the chemokine receptor CXCR4, whose expression on monocytes peaks at ZT13 (79). Decreased expression of surface CXCR4 abrogates rhythmic diurnal oscillations in monocytes, and has also been shown to affect their homing ability to peripheral organs such as the liver and lung (80). Myeloid deletion of *Bmal1* ablates the rhythmic trafficking of monocytes between bone-marrow, blood and peripheral organs, highlighting the importance of intrinsic monocyte clocks in this process (22). Inflammatory monocyte chemotaxis is also dependent on chemokine signaling through the CCL2:CCR2 axis (81). BMAL1 has been shown to control circadian monocyte trafficking by inhibiting the transcription of the chemokines *Ccl2* and *Ccl8* through recruitment of the polycomb repressive complex 2 (PRC2) (22). Monocyte release into circulation, as well as their eventual infiltration into organ tissues, is clearly dependent on the circadian expression of receptors and chemokines (82).

The transition of inflammatory monocyte from blood into peripheral tissues is also dependent on the leukocyte adhesion cascade. This process involves direct interactions between endothelial vascular cells and infiltrating leukocytes (83). At the start of this process, chemokines are released into the blood from tissue-resident cells. These activate receptors on circulating monocytes that boost the expression of adhesion molecules to facilitate trans-endothelial migration. The molecular clock in monocytes is crucial in this regulation. Deletion of *Bmal1* in monocytes results in increased expression of CD18 integrin (a transmembrane receptor facilitating extracellular matrix adhesion), decreased chemokine receptor CCR2, and loss of rhythm in gene expression of L-selectin (a homing receptor that aids binding to endothelial cells). The ultimate consequence of these changes in adhesion and chemokine receptors, is disruption of monocyte trafficking to target tissues (80). Adrenergic nerves of the autonomic nervous system are also important in governing leukocyte recruitment to tissues in a circadian manner through adrenoreceptor signaling in endothelial cells, which leads to increased expression of ICAM1 (an adhesion molecule that aids in transmigration of immune cells) (78). Circadian expression of endothelial ICAM1 and VCAM1 in the lungs and liver, which peak at ZT13, coincides with maximum leukocyte recruitment, and this peak at ZT13 in endothelial ICAM1 and VCAM1 is ablated with *Bmal1* deletion (80). Deletion of *Bmal1* in endothelial cells ablates the rhythmic migration of all leukocyte subsets, including inflammatory monocytes. Unlike adrenergic signaling, glucocorticoid signaling by the adrenal gland is not required for circadian trafficking of monocytes to organs such as the spleen (23).

Diurnal monocyte trafficking corresponds to the immune response to *Listeria*. Intraperitoneal infection at ZT8 results in lower counts of colony forming units (CFU), but increased lethality compared to infection at ZT0 (22). Myeloid deletion of *Bmal1* results in greater lethality to *Listeria* infection. This indicates that BMAL1 prevents an overactive and lethal immune response to *Listeria* by dampening monocyte trafficking. Indeed, myeloid *Bmal1* deletion also results in greater numbers of inflammatory monocytes in circulation and in the spleen (22). Collectively, these data demonstrate the central role of

the molecular clock in controlling monocyte migration via chemokines, their receptors, and adhesion molecules, which is highly relevant to the immune response and to disease susceptibility and progression.

CONCLUSIONS

It is evident that the molecular clock exerts significant control over key functions of innate immunity, in particular monocytes and macrophages. However, our modern 24/7 lifestyles are at odds with this closely regulated endogenous 24-h system of control. This is leading to a global increase in the prevalence of circadian disruption in human health (84). An estimated 20% of the work force are shift workers who, due to their schedule, are at increased risk for obesity, diabetes, cardiovascular disease, and cancer (85, 86). Furthermore, at least 80% of us experience social jet lag (84), defined as misalignment between our body clocks and social behaviors (87). Shift workers have been shown to have altered numbers of immune cells such as monocytes (85) and altered rhythms in cytokine output (88)—and similar effects are likely to be present in all of us who experience significant social jetlag. It is conceivable that these circadian disruptions are an important link between lifestyle, behavior, and disease. Most of the studies discussed in this review used animal models of circadian disruption via jet lag/shift work models, or circadian gene deletion, to study their effects on inflammatory functions of monocytes and macrophages. These will help us to understand and explain key mechanisms in the pathogenesis of many human inflammatory disorders such as cardiovascular disease (89), asthma (90), rheumatoid arthritis (91), and potentially many others. Understanding the underlying molecular mechanisms by which the molecular clock controls metabolism, phagocytosis, pattern recognition, and inflammatory mediator production in monocytes and macrophages will help us to develop new tools and therapies for chronic disease. These will help us to manage the conflicting pressures of modern lifestyles, that have developed in recent decades, with the tightly controlled internal circadian system that has evolved over a much longer timescale.

AUTHOR CONTRIBUTIONS

GT and JO'S contributed equally in the research, writing, and creating of diagrams for this manuscript. OK aided in writing and critically evaluated the manuscript. AC provided ideas for and aided in writing and critically evaluated the manuscript. JE provided the layout for the manuscript, wrote and performed a critical evaluation of the manuscript, and provided final checks of the manuscript's quality. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the following awards: a Science Foundation Ireland [Career Development Award (17/CDA/4699)] and Marie Sklodowska-Curie Actions (MSCA) award to OK, and a Science Foundation Ireland [Career Development Award (17/CDA/4688)] and an Irish Research Council Laureate Award (IRCLA/2017/110) to AC.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 14 August 2020
doi: 10.3389/fimmu.2020.01783

Circadian Host-Microbiome Interactions in Immunity

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The gut microbiome plays a critical role in regulating host immunity and can no longer be regarded as a bystander in human health and disease. In recent years, circadian (24 h) oscillations have been identified in the composition of the microbiota, its biophysical localization within the intestinal tract and its metabolic outputs. The gut microbiome and its key metabolic outputs, such as short chain fatty acids and tryptophan metabolites contribute to maintenance of intestinal immunity by promoting barrier function, regulating the host mucosal immune system and maintaining the function of gut-associated immune cell populations. Loss of rhythmic host-microbiome interactions disrupts host immunity and increases risk of inflammation and metabolic complications. Here we review factors that drive circadian variation in the microbiome, including meal timing, dietary composition and host circadian clocks. We also consider how host-microbiome interactions impact the core molecular clock and its rhythmic outputs in addition to the potential impact of this relationship on circadian control of immunity.

Keywords: microbiome, circadian, immunity, diurnal, short chain fatty acids

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Eleni Beli, Queen's University Belfast, United Kingdom
Christoph Reinhardt, Johannes Gutenberg University Mainz, Germany

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *03 April 2020*
Accepted: *03 July 2020*
Published: *14 August 2020*

Citation:
Butler TD and Gibbs JE (2020) Circadian Host-Microbiome Interactions in Immunity. Front. Immunol. 11:1783. doi: 10.3389/fimmu.2020.01783

INTRODUCTION

The circadian clock is a critical regulator of immunity, including homeostatic processes and responses to immune challenge (1–3). It is now very well established that intrinsic clocks within cells of the immune system regulate their function, influencing the manner in which they respond to a pathogen. However, extrinsic rhythmic signals also drive rhythmic behavior in immune cells. The gut microbiome is recognized as a major influence over the immune system, impacting on its development and daily function. Work over the last decade has established that this bacterial community exhibits 24 h oscillations in composition, biophysical localization and function. Furthermore, these oscillations are critical for driving rhythmic metabolic processes both within the gut and further afield in organs such as the liver. Here we examine the potential impact of circadian rhythms in the microbiome on immunity, and pose the question might rhythms in the microbiome influence circadian control of immunity?

The Circadian Clock

Entrainment to the 24 h environment is vital to allow organisms to temporally arrange their daily functions (such as feeding, metabolism and sleeping) to align with optimal conditions. Nearly every cell of the body (including those of the immune system) contains the molecular machinery required to keep the clock ticking (4). However, organisms cannot set the time correctly without the help of environmental cues termed "zeitgebers" (meaning time-givers). The most important zeitgeber is the light-dark cycle, which entrains the central clock within the suprachiasmatic nucleus (SCN).

Other zeitgebers, such as food availability (5), hormones (6), and body temperature (7) are also important, particularly for influencing peripheral pacemakers (8). Peripheral clocks are synchronized by the SCN, but continue to oscillate when uncoupled from the central clock, highlighting their ability to respond autonomously to changes in the local micro-environment (3, 9, 10).

The molecular clockwork machinery relies on transcriptional-translational feedback loops (TTFL), the duration of which dictates the period of the circadian rhythm. The primary TTFL involves activator proteins circadian locomotor output cycles kaput (CLOCK) and brain muscle arnt-like 1 (BMAL1) interacting with repressor proteins PERIOD and CRYPTOCHROME (4). CLOCK and BMAL1 heterodimerise and bind Enhancer-box (E-box) sequences on target genes for PERIOD and CRYPTOCHROME, which accumulate in the cytoplasm before translocating to the nucleus to inhibit further CLOCK-BMAL1 activity by negative feedback (11). The main TTFL is stabilized by an auxiliary feedback loop involving REV-ERBα and retinoid-related orphan receptor (ROR)α, which repress and activate BMAL1, respectively (12). In this review, we consider bi-directional interactions between the circadian timing system and the gut microbiome and how this influences immune function.

The Microbiome

The microbiome is defined as an environment containing a heterogeneous community of microbes (including bacteria, fungi, and viruses) and the functional consequences of these microbes, such as their metabolic outputs and interaction with the host. By contrast, microbiota solely describes the community of organisms in isolation (13). It is generally accepted that the fetus develops in a sterile environment and first contact with microbes occurs during birth. An infant's microbiome is influenced by delivery route and method of feeding (14). The first 2–3 years of life oversee significant flux in the composition of the gut microbiome before assuming an adult-like profile, influenced by cessation of breast-feeding, increasing complexity of dietary nutrients and environmental exposures, including antibiotics (15, 16).

Abbreviations: 5-HIAA, 5-hydroxyindole-3-acetic acid; AHR, Aryl hydrocarbon receptor; AMP, Antimicrobial peptide; ARNT, Aryl hydrocarbon receptor nuclear translocator; BA, Bile acids; BMAL1, Brain muscle arnt-like 1; CLOCK, Circadian locomotor output cycles kaput; CNS, Central nervous system; EAE, Experimental autoimmune encephalomyelitis; FFAR, Free fatty acid receptor; FMT, Fecal microbial transfer; GF, Germ free; GM-CSF, Granulocyte macrophage colony stimulating factor; GPCR, G-protein coupled receptor; HCAR, hydroxycarboxylic acid receptor; HDAC, Histone deacetylase; HFD, high-fat diet; HIF, Hypoxia-inducible factor; I3A, Indole-3-acetate; IAId, Indole-3-aldehyde; IEC, Intestinal epithelial cell; IL, Interleukin; ILC, Innate lymphoid cell; IPA, Indole 3-proprionic acid; isoDCA, isodeoxycholic acid; LCA, lithocholic acid; LPS, lipopolysaccharide; NFIL3, nuclear factor interleukin 3 regulated; NOD, non-obese diabetic; OTU, Operational taxonomic unit; PAS, Per/Arnt/Sim; PPAR, peroxisome proliferator activated receptor; PXR, Pregnane X receptor; ROR, Retinoid related orphan receptor; SCFA, Short chain fatty acid; SCN, Suprachiasmatic nucleus; SFB, Segmented filamentous bacteria; STAT, Signal transducer and activator of transcription; TCCD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; T_h17, T helper 17; Treg, Regulatory T cell; TLR, toll-like receptor; TTFL, Transcriptional-translational feedback loop.

The advent of high throughput genome-sequencing technology and large scale projects such as the National Institute for Health's Human Microbiome Project (17) and the European Commission's Metagenomics of the Human Intestinal Tract (18) have dramatically improved our understanding of the phylogenic composition and variability of the microbiota, which in humans has over 150 genera from three main phyla: Firmicutes, Bacteroidetes and Actinobacteria (19). The abundance of these phyla varies significantly between individuals (20, 21).

Our understanding of microbiome function is growing constantly, with main areas of interest in development, immunity and metabolism. Here we focus on the impact of gut microbiota rhythmicity in directing immunity.

The Gut Microbiome Shapes Immunity

The gut microbiota contribute to the development, maturation and regulation of the host immune system. The importance of the microbiome in shaping the immune system is perhaps best demonstrated by studies with germ free (GF) animals. GF mice have underdeveloped gut-associated lymphoid tissues, fewer and smaller Peyer's patches and mesenteric lymph nodes, and impaired development of isolated lymphoid follicles (22, 23). GF mice have reduced levels of secretory IgA in the intestine (24), and the morphology of the intestinal epithelial cells (IECs), which are normally in direct contact with the microbiota, is modified, with altered microvilli formation and slower cell turnover (25). Furthermore, the absence of a microbiota is associated with arrested capillary network formation within the intestines (26, 27).

Studies with GF mice elegantly demonstrate that commensal bacteria are essential for the development of immune cell subsets, both within the gut lamina propria and further afield. The absence of gut commensals leads to defects in circulating innate immune cell populations (including neutrophils, monocytes and macrophages) and cells within systemic immune sites (including the spleen, bone marrow and liver) as well as delayed neutrophil aging (28–30). Much focus in the field has been surrounding the influence of the microbiota on shaping T cell populations, although it is clear that this influence extends much further to include B cells and innate immune cells. Work over the last decade has assigned roles for individual commensal species in influencing the composition of the lamina propria T lymphocyte subsets. For example, in rodents, segmented filamentous bacteria (SFB) induce intestinal T helper 17 (T_h17) cells (31, 32), and in humans *Bifidobacterium adolescentis* plays a similar role (33). GF mice have reduced numbers of regulatory T cells (Tregs) in the lamina propria, which can be rescued by re-colonization with strains of *clostridium* (34). These studies highlight the importance of the microbiota for maintenance of the T_h17/Treg axis. In support, outcomes of mouse models of autoimmunity are often dependent on colonization status. For example, GF mice exhibit marked attenuation in murine models of experimental arthritis (35), experimental autoimmune encephalomyelitis (EAE) (36) and uveitis (37). In the case of EAE (a model of CNS inflammation), the absence of the microbiota is associated with loss of susceptibility as a consequence of perturbations in the balance of Tregs and T_h17 cells. This balance

is restored after re-colonization with SFB and susceptibility returns (36). Similarly the reduction in T_h17 cells associated with GF mice results in an attenuated arthritis in the K/BxN model of experimental arthritis (35). Intriguingly, in uveitis, retina-specific T cells have been detected in the intestine. Given that the eye is immunologically privileged, it may be that the gut microbiome is playing a role in antigen-mimicry, whereby T cells are triggered by a surrogate antigen present in the gut environment, mimicking a retinal antigen (38). The existence of a gut-retina axis is supported by the presence of uveitis in a subset of patients with inflammatory bowel disease, although further work is required to implicate the microbiome directly (39).

Murine models of autoimmune-driven Type 1 diabetes mellitus, such as non-obese diabetic (NOD) mice are driven in part by activation of innate immune component toll-like receptors (TLR) (40). NOD mice with genetic deletion of *MyD88*, a common adaptor protein for TLR signaling, are protected from type 1 diabetes, but this protection is lost in GF conditions (41). In keeping, NOD mice treated with antibiotics from an early age demonstrate an increased incidence of type 1 diabetes mellitus (42, 43). Together, these data suggest the microbiome modulates innate immunity through TLR signaling to impact risk of autoimmune metabolic diseases such as diabetes in mice.

Metabolic Outputs of the Microbiota Direct Immunity

Many of the effects the microbiome has on immunity are attributable to the metabolic outputs of the gut microbiota (44, 45). Intestinal bacteria produce a wide range of metabolites, including short chain fatty acids (SCFAs), tryptophan metabolites, essential vitamins, phenolic acids, polyamines and bile acids, which act to modulate host metabolism and immunity (**Figure 1**).

Short Chain Fatty Acids

SCFAs [for example, acetate (C_2), proprionate (C_3), and butyrate (C_4)] are volatile fatty acids composed of a backbone of between 1 and 6 carbon atoms. Derived from bacterial anaerobic fermentation of dietary fibers in the colon, they promote intestinal epithelial barrier function and regulate the host mucosal immune system. SCFAs are important for maintenance of IEC turnover and barrier function (46–48). Butyrate is a critical source of energy for IECs (49), and also regulates expression of the transcription factor *hypoxia-inducible factor* (*HIF)1α*, which acts to co-ordinate barrier function (46) and induces IL10RA, which promotes expression of the tight junction protein Claudin 2 (48). In addition, SCFAs act on multiple gut resident immune cells to facilitate maintenance of intestinal immunity. The earliest observation of the role for SCFAs in maintenance of immune cell populations was made in Tregs. Butyrate and proprionate facilitate extrathymic generation of Tregs (50, 51) by enhancing histone H3 acetylation in the promoter and conserved non-coding sequence regions of the FoxP3 locus (52). More recently it has been established that the effects of SCFA extend far beyond these anti-inflammatory cells to influence the function of both adaptive and innate immune cell populations. These include macrophages (53, 54), B cells (55), $CD8^+$T cells (56), type 3 innate lymphoid cells (ILC3s) (57, 58) and neutrophils (58). For example, butyrate alters the metabolic behavior of intestinal macrophages, promoting a state of alternative activation and thus promoting microbial tolerance (53).

Tryptophan Metabolites

Tryptophan is a dietary essential amino acid metabolized in the gastrointestinal tract via three different pathways: the kynurenine pathway; the serotonin pathway; or via direct metabolism by microbiota (59). Whilst the former two metabolic pathways are regarded as host-dependent, the microbiota has been shown to influence them (59). Tryptophan metabolism by the microbiota includes the transformation of tryptophan to indole and its derivatives, many of which are ligands for the aryl hydrocarbon receptor (AhR). This includes: indole-3-aldehyde (IAId), indole-3-acetate (I3A), indole 3-proprionic acid (IPA), indole-3-acetaldehyde (IAAId), 5-hydroxyindole-3-acetic acid (5-HIAA) and indoleacrylic acid.

AhR is a Per/Arnt/Sim (PAS) domain protein and a fundamental modulator of immunity, controlling the differentiation and inflammatory potential of innate and adaptive immune cells at the gut barrier and systemically (60, 61). AhR shares significant sequence homology to the core clock protein CLOCK and as such offers itself as a nodal point between the gut microbiota, circadian clocks and immunity (**Figure 2**). Ligand bound AhR heterodimerises with aryl hydrocarbon receptor nuclear translocator (ARNT) in the nucleus, but can also heterodimerise with BMAL (62) to disrupt normal binding of CLOCK/BMAL to the *Per1* promoter (63). AhR plays a role in regulating circadian rhythms in behavior and physiology, as loss of one allele alters responses to changes in the light dark cycle, increases the amplitude of hepatic core clock genes and alters rhythms in glucose and insulin (64). Conversely, AhR activation alters rhythmic expression of core circadian regulators within the periphery; for example chronic administration of the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dampens rhythmic expression of core clock genes and clock-controlled hepatic genes disrupting circadian regulation of hepatic metabolism (65). To date there is no direct evidence that microbiota-derived tryptophan metabolites affect timing mechanisms, but this is a certainly a possibility.

Tryptophan metabolites produced by the microbiota are important for regulation of immunity. For example, IAId produced by lactobacilli induces IL22 production to support antifungal resistance and ameliorate colitis in mouse models (66). I3A reduces the production of pro-inflammatory cytokines by macrophages and slows their migration toward a chemotactic signal in an AhR dependent manner (67). Additionally, tryptophan metabolites such as IPA act via pregnane X receptors (PXR) to regulate intestinal barrier function through TLR4 activity (68, 69). Recent work has uncovered a mechanism whereby bacteria derived butyrate enhances production of 5-HIAA which acts via AhR to support regulatory B cell function (70). This highlights the importance of interactions between different microbial species to promote immunity.

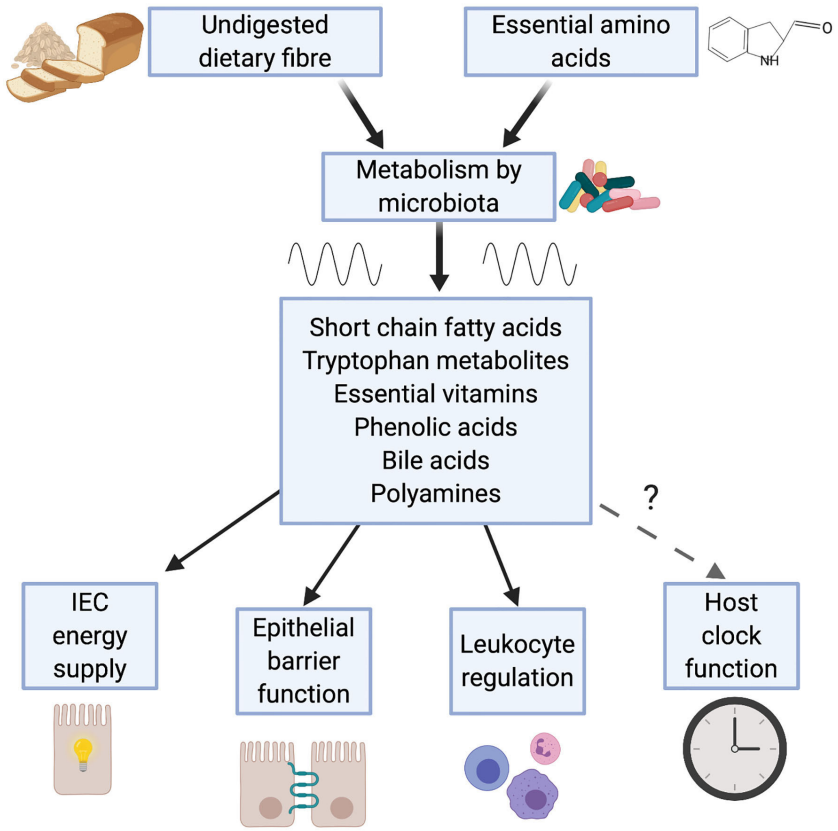

FIGURE 1 | Metabolic outputs from the microbiome influence immunity. Bacteria within the gut breakdown substances derived from the diet. Emerging evidence demonstrates that production of a number of these metabolites is rhythmic, a consequence of feeding rhythms and rhythmic function of the microbiome. Many of these microbial metabolites (e.g. short chain fatty acids, tryptophan metabolites and bile acids) play a key role in immunity, contributing to: intestinal epithelial cell (IEC) function; maintenance of leukocyte populations such as regulatory T cells and macrophages and intestinal tolerance.

Bile Acids

Bile acids (BAs) are cholesterol-derived molecules produced in the liver and secreted into the duodenum. Whilst the vast majority (~95%) are re-absorbed through the terminal ileum, several different phyla of gut bacteria can transform the remainder to secondary bile acids, with immune modulatory effects. Recently there has been a flurry of activity in this field ascribing a role for secondary BAs in T cell biology. The lithocholic acid (LCA) derivatives 3-oxoLCA and isoalloLCA regulate T_h17 and Treg cell differentiation in the lamina propria (71) and RORγ^+ Tregs in the colon (72). Furthermore, isodeoxycholic acid (isoDCA) promotes the generation of peripheral Tregs (73).

Evidence for Intrinsic Circadian Clocks Within Gut Bacteria

The existence of intrinsic circadian clocks has rarely been demonstrated in organisms outside of the eukaryotic kingdom, with the exception of certain photosynthetic cyanobacteria. *Synechococcus elongatus* utilizes 3 clock proteins (KaiA, KaiB and KaiC) in a post-translational oscillator cycle of phosphorylation and de-phosphorylation to generate 24 h rhythms (74). The *S. elongatus* clock can synchronize to 24 h temperature cycles and directs clock-regulated gene expression of almost the entire genome (75, 76). It appears that it is not necessary to have all three Kai proteins in order to exhibit rhythmicity. *Rhodobacter sphaeroides* are purple photosynthetic bacteria which express self-sustaining rhythmicity under aerobic condition but lack *KaiA* (77), suggesting that either KaiB and KaiC function differently from those in Cyanobacteria, or that there may be novel components regulating the clock mechanisms in place here.

The existence of timekeeping components in non-photosynthetic bacteria has been addressed in part by exploring whether homologs of the *Kai* genes exist in other bacteria. Whilst *KaiB* and *KaiC* are quite highly conserved, *KaiA* is missing in many other prokaryotes (78). However, like *R. Sphaeroides*, circadian cycles can still be still displayed as in the case of the marine cyanobacterium *Prochlorococcus* (77). *Enterobacter aerogenes* is the first gut commensal bacteria found to express 24 h rhythms outside the host (79). This gram-negative motile bacterium from the phylum Proteobacteria shows circadian rhythmic swarming behavior in the presence of melatonin, which was unreproducible in other gram-negative bacteria such as *Escherichia coli* or *Klebsiella pneumoniae*. Furthermore,

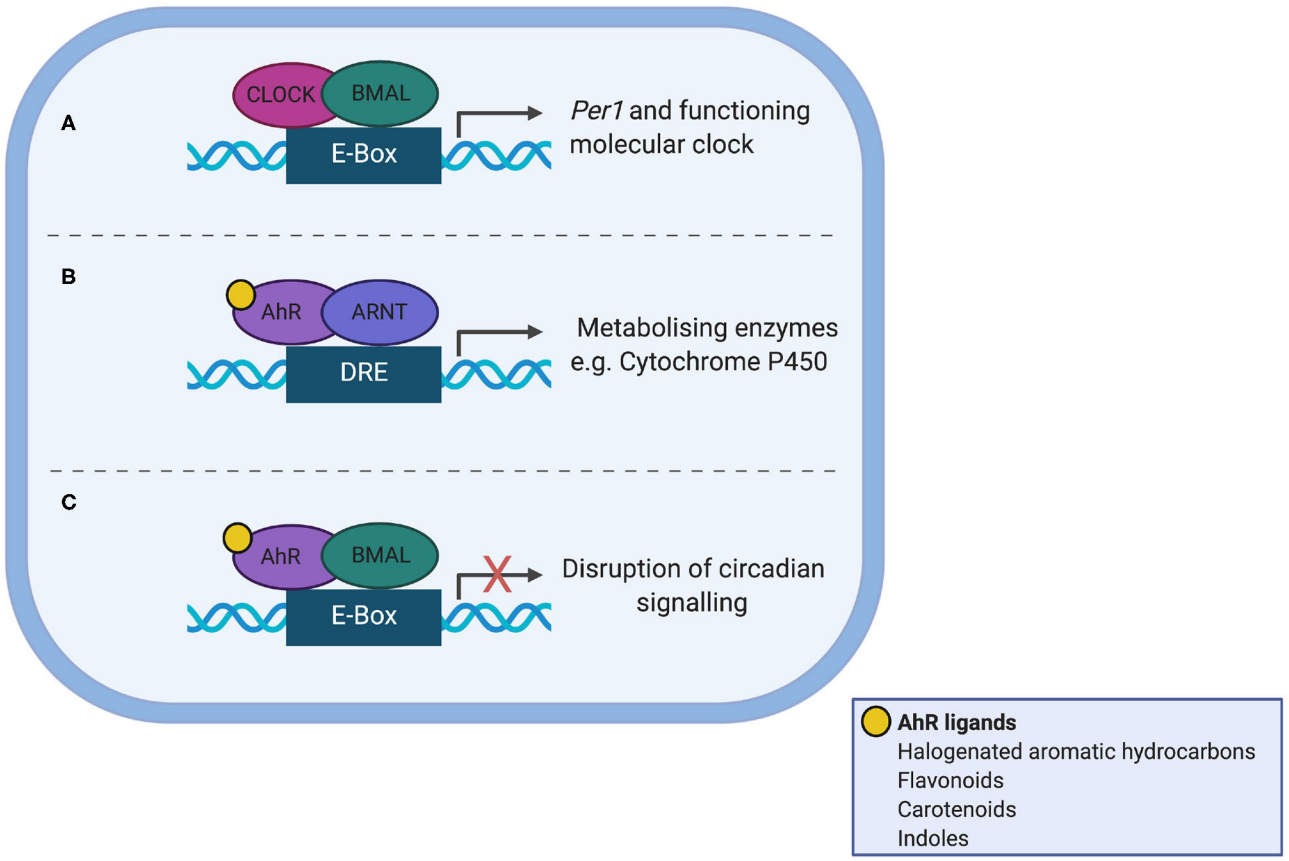

FIGURE 2 | Interaction between the aryl hydrocarbon receptor (AhR) and the molecular clock. **(A)** In normal circadian clock function, CLOCK and BMAL heterodimerise and bind the E-box promotor, to transcribe *Per1*. **(B)** AhR shares sequence homology with CLOCK. Ligand-bound AhR heterodimerises with aryl hydrocarbon receptor nuclear translocator (ARNT) in the nucleus leading to transcription of enzyme superfamilies. **(C)** Ligand-bound AhR can also heterodimerise to BMAL1, which disrupts normal binding of CLOCK/BMAL to the *Per1* promoter, leading to circadian disruption.

transformation with a flagellar motor-protein-driven luciferase reporter reveals temperature-compensated circadian rhythm of luciferase activity synchronized by melatonin (80). Given the abundance of extrapineal melatonin secreted within the gut (81), these studies present this diurnal hormone as a potential zeitgeber for the gut microbiome and may present one mechanism of host control of microbiome rhythmicity. Further studies are required to understand the intrinsic circadian mechanisms of *E. aerogenes* and explore the presence of intrinsic rhythms within other species of the gut microbiota. One possibility is that gut commensals have evolved to respond rhythmically to environmental signals through horizontal gene transfer with human hosts (80, 82). The presence of intrinsic circadian machinery in gut microbiota would have far-ranging implications for understanding host-microbiome interactions.

Rhythmicity of the Microbiome

With the discovery of resident intestinal clocks (83–85) and rhythmicity in multiple immune cells, interest is growing in the circadian influence on host-microbiome interaction and immunity. 16S rRNA gene sequencing of the fecal microbiome has demonstrated diurnal oscillations in the relative abundance of roughly 15% of OTUs, accounting for around 60% of the murine microbiome, with corresponding oscillation in 10% of OTUs in humans (86, 87). In addition rhythmicity in total microbiota has been described, driven predominantly by Bacteroidetes, which peak during the murine active phase (88). Diurnal oscillations in abundance also exist robustly in healthy humans despite marked inter-human variation in gut microbiome composition (20).

The potential functional consequences of a rhythmic microbiome have been highlighted via metagenomic studies showing oscillations in up to a quarter of total microbiome genetic material in both mice and humans (86). KEGG pathway analysis identified time-of-day grouping of functions such as cell growth and energy metabolism during the murine dark phase, with environmental sensing and flagellar assembly during the light phase (86). Human metagenome analysis demonstrated inversion of peaks in pathway activity compared to nocturnal mice (86). Interestingly, in a cohort of patients with Type 2 diabetes mellitus, diurnal oscillations in microbiome composition were dampened with metagenomic analysis

highlighting impact on microbial pathways that process amino acids and fatty acids (89). This is notable as one of the first studies to associate an arrhythmic microbiome with a human disease state.

Diurnal oscillations have been detected in the biophysical distance between mucosal bacteria and IECs, with closest proximity during the murine active, dark phase (90). In mice with global knockout of RegIIIγ, an antimicrobial peptide (AMP) secreted by IECs into the mucus layer, rhythms in the mucosal microbiome were attenuated, suggesting AMPs have a part to play in control of mucosal rhythmicity as well as epithelial cell-microbe distance (90, 91). The mucosal microbiome in closest proximity to the epithelial barrier is most likely to be sampled by mucosal immune components and co-ordinated rhythmicity between the host and microbiome will likely produce the most effective balance in gut homeostasis between tolerance and activated immune response. Thus, whilst the fecal microbiome is easy to sample, the mucosal microbiome may provide more answers to the links in circadian host-microbiome interactions.

Metabolic outputs of the microbiota also exhibit diurnal rhythmicity (**Table 1**). This is heavily influenced by feeding rhythms of the host providing targets for metabolism, but rhythms in microbial activity clearly also contribute. SCFA levels show diurnal fluctuations in concentration in feces, caecal samples and plasma (92–94). These oscillations are regulated by feeding time as rhythmicity in caecal SCFA are abolished in arrhythmic *Bmal1*$^{-/-}$ mice, but restored by a restricted feeding regimen (94). Oscillations are also sensitive to dietary composition as a high-fat diet (HFD) abolishes rhythms in fecal butyrate (92). While levels of liver-derived conjugated bile acids exhibit post-prandial fluxes during the day in humans, levels of unconjugated bile acids peak late at night, likely reflecting their dependence on microbial activity (95). Many other microbiota-derived or microbiota-modulated metabolites exhibit diurnal rhythms in the intestines and serum, including the polyamine ornithine and the amino acid proline (90, 96). To date, the impact of these rhythms on downstream immunological processes has not been explored, but the importance of microbial metabolites in host immunity makes this an important area to explore.

Timing of food intake is a circadian process centrally controlled by the host and influenced by hunger, food availability and social and cultural norms (97). Mice with disrupted circadian light entrainment show a loss of fecal microbiome diversity, loss of microbiome oscillations, increased weight gain and impaired glucose tolerance despite similar calorie intake and energy expenditure to controls on a standard light:dark cycle (98–100). In a "jet-lag" model, mice phase-shifted by 8 h every 3 days for 4 weeks demonstrate attenuated diurnal oscillations in microbiome abundance, likely driven by loss of diurnal food intake (86).

In addition to timing of food, dietary composition also impacts the microbiome rhythmicity. The availability of high-fat diet *ad libitum* drives mice to lose their diurnal eating habits and spread their intake through the day (101). This dampens diurnal oscillations in the microbiome (which are restored when time-restricted feeding is applied) and results in persistent microbial dysbiosis (87). Jet-lagged mice fed a high-fat diet experience greater weight gain compared to non-shifted controls, despite equal food intake; a phenotype attenuated by concurrent antibiotic administration or provision of a high-fat diet to GF mice (86, 92). Fecal microbial transplantation (FMT) from mice with jet lag to GF, non-shifted mice conferred an obesity phenotype not seen in FMT of non-jet-lagged mice (86). This suggests that hosts with a disrupted clock are more susceptible to harboring a dysbiotic microbiome, which is sufficient to drive adverse metabolic consequences for the host (**Figure 3**).

TABLE 1 | Microbial metabolites with demonstrated daily rhythmicity in tissue or circulating concentrations.

Metabolite	Species	Tissue	References
Short chain fatty acids			
Butyrate	Mouse	Feces	(92)
	Mouse	Feces	(93)
	Mouse	Plasma	(94)
	Mouse	Caecum	(94)
Acetate	Mouse	Feces	(93)
	Mouse	Plasma	(94)
	Mouse	Caecum	(94)
Proprionate	Mouse	Feces	(93)
	Mouse	Plasma	(94)
Bile acids			
Unconjugated Bile acids	Human	Serum	(95)
Amino Acids			
Threonine	Mouse	Serum	(90)
Ornithine	Mouse	Serum	(90)
Proline	Mouse	Serum	(90)
α-aminobutyric acid	Mouse	Serum	(90)
Vitamins			
Biotin	Mouse	Caecum	(90)
Lactate	Mouse	Caecum	(90)
Polyamines			
Spermidine	Mouse	Serum	(96)

The Host Clock Shapes the Microbiome

Whilst feeding times clearly drive rhythms in the microbiome, the host circadian molecular clock also plays a role, with studies evidencing the importance of the biological clock in regulating the composition of the microbial community as well as the rhythmicity. Studies utilizing *Clock*$^{\Delta 19}$ mice (as a genetic approach to model circadian disruption) report increased intestinal hyperpermeability (102) and reduced microbial diversity (103) of the gut microbiome. It is feasible, but as yet undemonstrated, that these changes to the microbiota influence barrier integrity (or *vice versa*). To address the contribution of the host clock in driving microbiome rhythmicity, studies often use transgenic mice lacking a functional clock. Most studies here have been undertaken using animals with global deletion of core clock genes, which complicates interpretation of results as this likely leads to arrhythmic feeding behavior. Mice with global *Bmal1* knockout are arrhythmic and demonstrate attenuated compositional microbiome rhythmicity, whilst

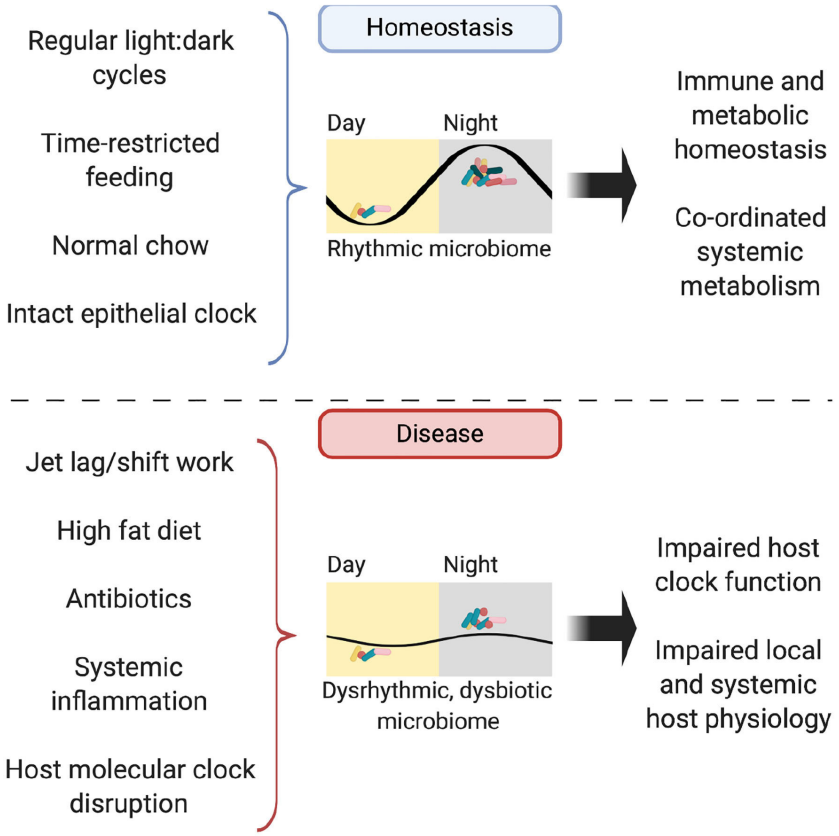

FIGURE 3 | The rhythmic microbiome. In homeostasis, the microbiome is rhythmic in its composition, abundance and biogeographic distance from the intestinal mucosa, with its abundance peaking in the murine dark, active phase. Possible entraining factors include light:dark cycles, timing and composition of food intake and host molecular clock function. Functioning oscillations in the microbiome drive homeostasis in both immunity and metabolism, both locally in the intestine and further afield in organs such as the liver. In disease, disruption to entrainment via jet lag, high fat diet, illness and treatments such as antibiotics lead to perturbations in microbiome rhythmicity and subsequent impairment of local and systemic immune-metabolic homeostasis.

retaining circadian oscillations in total fecal microbiome abundance (88). Arrhythmic mice with global *Per1/2* deletion lose rhythmicity in abundance of fecal and mucosal microbiomes as well as metagenomic and metabolic outputs (86, 90). In addition, the intestinal microbiome of global *Per1/2* knockout mice is dysbiotic with less diversity compared to littermate controls (86). Instigation of a time-restricted feeding schedule to *Per1/2* knockout mice to align food consumption with wildtypes recovers oscillations in the microbiome (86). Targeting the molecular clock in specific cells will further delineate the relative contributions of central host rhythms and circadian rhythms in local mucosal immunity to microbiome dysbiosis. Indeed, this approach has revealed a subset of ILCs as critical regulators of the rhythmic microbiome.

ILCs as Rhythmic Regulators of the Microbiome

ILCs are circadian rhythmic tissue-resident lymphoid cells. There are currently five subsets of ILCs, which align with their T helper cell counterparts comprised of natural killer cells, ILC1s, ILC2s, ILC3s and lymphoid tissue inducer cells (104). ILC3s are innate counterparts to T_h17 cells, characterized by expression of RORγt and production of IL17, IL22, and GM-CSF and are the predominant ILC class found at mucosal sites such as the intestine. ILC3s regulate the composition of the microbiota by encouraging preferential commensal growth whilst contributing to removal of pathogens. ILC3-derived IL22 binds directly to IECs and acts through Signal Transducer and Activator of Transcription (STAT)3 to support a multitude of functions that bolster the luminal biophysical barrier including production of AMPs, goblet cell differentiation and subsequent mucus production (105, 106). In addition, IL22 improves colonization resistance by fucosylating IEC-associated proteins that commensal bacterial preferentially use as an energy source, thus helping commensals out-compete pathogens (107, 108).

Recent work has uncovered the importance of the circadian clock on the function of ILC3s (**Figure 4**). In homeostasis, ILC3s exhibit oscillating expression of core clock genes, transcription factors (such as *Ahr)* and functional genes (such as *il17* and *il22)* (109–111). ILC3-specific knockout of either *Bmal1* or *Rev-erbα* decreases intestinal ILC3 numbers, decreases gene expression of anti-microbial peptides and mucus production and disrupts oscillations of Proteobacteria and Bacteroidetes

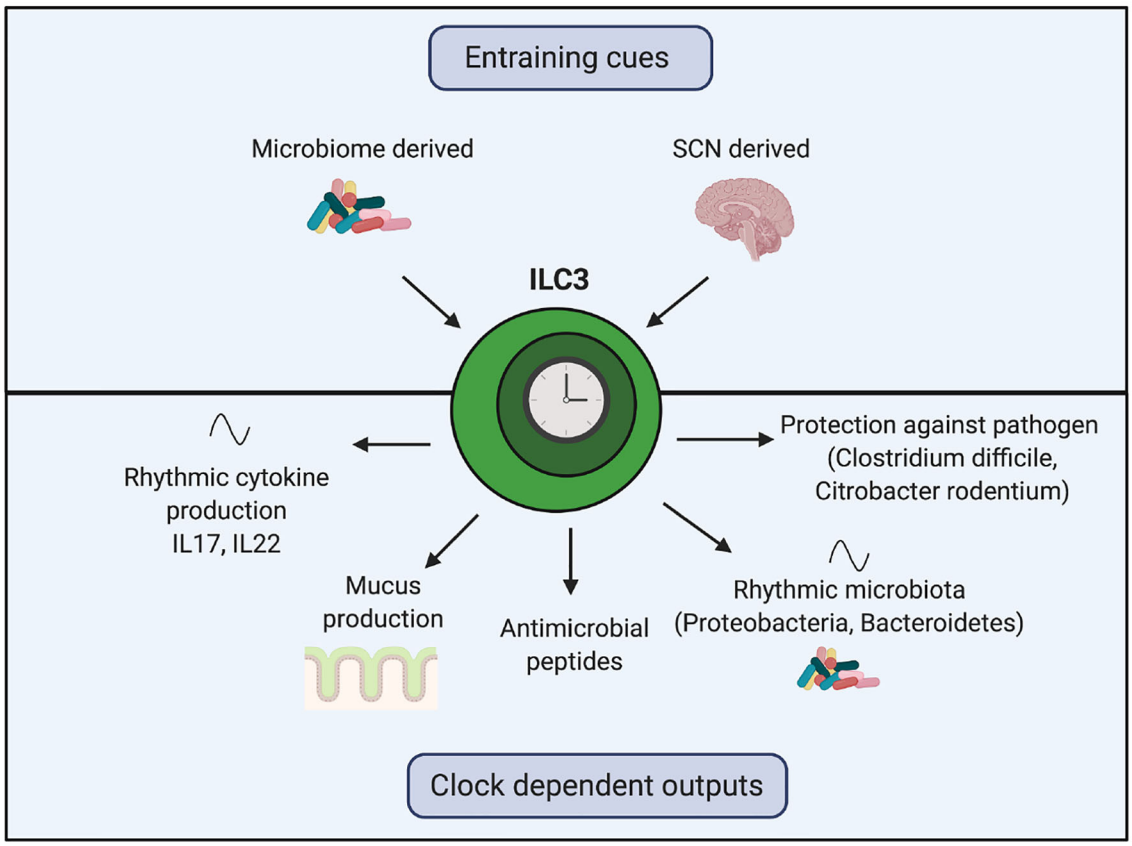

FIGURE 4 | The role of the ILC3 clock in regulating the rhythmic microbiome and barrier function. Clocks within ILC3s are synchronized by the SCN and local microbiome-derived cues. Genetic manipulation of core clock genes within ILC3s demonstrates the importance of this intrinsic clock for regulating crosstalk between the host and the microbiome. Loss of the ILC3 clock results in decreased gene expression of anti-microbial peptides, reduced mucus production, disrupted oscillations in components of the microbiota and cytokines and impaired responses to harmful bacteria.

in the microbiome (109–111). Intriguingly, these genetic manipulations do not affect abundance of ILC3s in the spleen and lung, suggesting intestinal ILC3s may be uniquely reliant on *Bmal1* (109).

Mice with *Rev-erbα*-deficient ILC3s exposed to *Clostridium difficile* show an exaggerated IL17 response and a higher bacterial burden (111). Overproduction of IL17 and IL22 in *Bmal1*-deficient ILC3s is abrogated by antibiotic treatment (110). Lymphocyte-deficient mice with *Bmal1*-deficient ILC3s are more susceptible to gut inflammation induced by *Citrobacter rodentium*, with increased epithelial barrier permeability and reduced survival (109). In wildtype mice, oral antibiotic treatment shifts the timing of *Per1* expression in ILC3s and dampens IL22 oscillations (109, 110). Together, these observations illustrate the importance of the ILC3-intrinsic circadian clock in regulation of crosstalk between host ILC3s and the microbiome. In humans with inflammatory bowel disease, there is a reduction in ILC3s within inflamed intestine compared to intra-individual non-inflamed tissue, with associated disruption of ILC3 circadian gene expression (110). Of interest, *Rev-erbα* gene expression was elevated in human ILC3s from inflamed tissue (110, 112). These perturbations to the ILC3 clockwork may have downstream effects on management of microbial rhythmicity.

Multiple components of mucosal immunity exhibit rhythmicity, including the microbiome, IECs, dendritic cells and ILC3s, which will likely need to be co-ordinated and entrained for maximal gut homeostasis. It will be pertinent to delineate the local power balance and determine which circadian clocks, local or central, drive rhythmicity in the gut microbiome during homeostasis and the consequences of disease (90, 113–115).

Rhythms in the Gut Microbiome Affect Rhythmic Processes Elsewhere

Communication between the host and microbiome is bi-directional. Whilst host clocks and rhythmic feeding drive daily variation in the composition of the microbiome, signals derived from the microbiome can influence circadian host physiology. We are now beginning to establish an understanding of how the microbiota and its metabolic outputs effects rhythmic processes.

A significant proportion of rhythmic transcripts within the intestines are influenced by the intestinal microbiota. The dependence of rhythmic expression of the core clock genes

on the microbiome appears to be tissue dependent, with depletion of the microbiome affecting clock genes such as *Rorα* and *Rev*-erbα differently in the colon compared to the small intestines. Mukherji et al. mapped the rhythmic transcriptome in ileal IECs, which includes *nuclear factor interleukin 3 regulated* (*Nfil3)*, *tlr* genes and clock genes *Rorα* and *Rev-erbα*. *D*epletion of the microbiome via administration of antibiotics results in the cessation of rhythmic gene expression and a fall in *Tlr* expression, whilst *peroxisome proliferator activated receptor* (*Ppar)α* becomes constitutively expressed, suppressing downstream circadian outputs including *Nfil3*, with subsequent impairment of host metabolism (113). Administration of LPS (as a surrogate for microbiome activation of toll-like receptors) reinstated RORα-driven circadian *Tlr* expression and recovered homeostatic IEC outputs including hypothalamic-pituitary-adrenal axis-independent corticosterone rhythmicity (113). NFIL3 is important in the development and regulation of immune cells including ILCs, macrophages and dendritic cells and may be one pathway for an arrhythmic microbiome to impact host immunity (116–118). In addition, lower NFIL3 levels have been detected in patients with active inflammatory pathologies such as colitis and arthritis, conditions that have been associated with microbiome dysbiosis (117, 119).

Transcriptomic examination of colonic IECs by Thaiss et al. demonstrated that depletion of the microbiota causes loss of rhythms in many pathways (including nucleotide metabolism and cell-cycle pathways) without affecting the core circadian clock genes (90). Intriguingly, this study also noted emerging *de novo* circadian rhythms in genes associated with metabolic pathways, suggesting the host may acquire compensatory oscillatory programmes in the absence of the microbiota. In keeping, depletion of the microbiome resulted in distinct changes to the temporal organization of the chromatin landscape within IECs. The regulation of transcriptional oscillations is dependent on rhythmic bacterial adherence, as mice lacking RegIIIγ (which have an abundant but non-rhythmic mucosal microbiome) showed an overlapping oscillating transcript with antibiotic-treated mice (90).

Further support for a role for the intestinal microbiota in programming daily rhythms in metabolic networks within the gut comes from a study, which found the gut microbiota drives rhythmic recruitment of HDAC3 to chromatin in IECs of the small intestine (120). Resultant HDAC3-NCoR complexes produce synchronized diurnal oscillations in histone acetylation, expression of metabolic genes, nutrient uptake and intestinal lipid absorption. *Bacteroides thetaiotaomicron* is critical here, as mono-colonization of GF mice with this Bacteroide is sufficient to restore rhythms in HDAC3 expression. Interestingly, the mechanism appears to be restricted to the small intestine; within the colon, genome wide acetylation rhythms persist in the absence of the microbiota (90, 120).

The microbiota also influences metabolic programmes within the gut via direct action on the clock. Wang et al. describe a signaling network by which the microbiota modulates clock activity with IECs to influence their metabolic programme (114) (**Figure 5**). This network integrates local immune cells which respond directly to the microbiota and signal to the IECs. Gram-negative bacteria are detected via toll-like receptors on myeloid cells within the intestinal lamina propria, via a number of potential routes (121). This prompts IL23 release which drives IL22 production by ILC3s. IECs respond to IL22 by up-regulating expression of STAT3, which binds to the promoter of *Rev-erbα* to repress expression. This reduction in REV-ERB levels allows enhanced transcription of *Nfil3*, a transcription factor that regulates a circadian transcription program of genes involved in lipid uptake, immunity and metabolism. In this way, the gut microbiota functions as an entraining cue for systemic processes and thus daily oscillations in the abundance of key bacterial species and/or the biogeography within the gut may be critical here. These studies highlight the ability of the local immune network to sense daily changes in the microbiota and raise the possibility that local immune networks may also be temporally responsive to the microbiota.

It is evident from these studies that the microbiota itself can modulate the core clockwork machinery. It is also now becoming clear that metabolic outputs from the microbiota can directly alter the phase and amplitude of the circadian clock. Oral administration of SCFAs can induce phase changes in peripheral clocks (122). Additionally, metabolites produced by *Clostridium sprogenes* such as [3-(4-hydroxyphenyl) propionic acid (4-OH-PPA) and 3-phenylpropionic acid (PPA)] increase the amplitude and lengthen the period of PER2 oscillations in organotypic SCN slices and other tissues (123). Polyamines (putrescine, spermidine and spermine) can regulate the circadian period of cultured cells and alter the circadian period of mice, likely as a consequence of their ability to promote the interaction between PER2 and CRY1 (96). Finally, secondary bile acids (deoxycholic acid and chenodeoxycholic acid) alter clock gene expression in the ileum, colon and liver (124). Given that some secondary bile acids have been shown to be able to directly bind to RORγt (71), this represents a potential mechanism by which bile acids may modulate the core clock. Together, these findings highlight the possibility that microbiome-derived metabolites act as zeitgebers, perhaps fine-tuning the clock to feeding rhythms.

In addition to the microbiota driving rhythms within the gut, bacterial-derived signals also act further afield to programme circadian networks in the liver (90, 125). The gut microbiome has been identified as a potential driver of HFD-induced changes in liver circadian metabolism (125). The nutritional challenge afforded by HFD drives transcriptional and metabolic reprogramming within the liver, resulting in a gain of rhythms in previously non-circadian transcription factors such as peroxisome proliferative activated receptor γ (PPARγ) (126, 127). Murakami et al. demonstrated that fecal transfer from HFD-fed mice to recipient normal chow fed mice is sufficient to elicit the emergence of circadian rhythms in PPARγ leading to specific rewiring of circadian transcription. Thaiss et al. demonstrated in mice fed normal chow, depletion of the microbiome re-programmes the liver transcriptome, with both loss and gain of rhythmic pathways (90). They went on to identify polyamines and amino acids as contributing microbiome-regulated rhythmic metabolites that signal from the gut to the liver to regulate

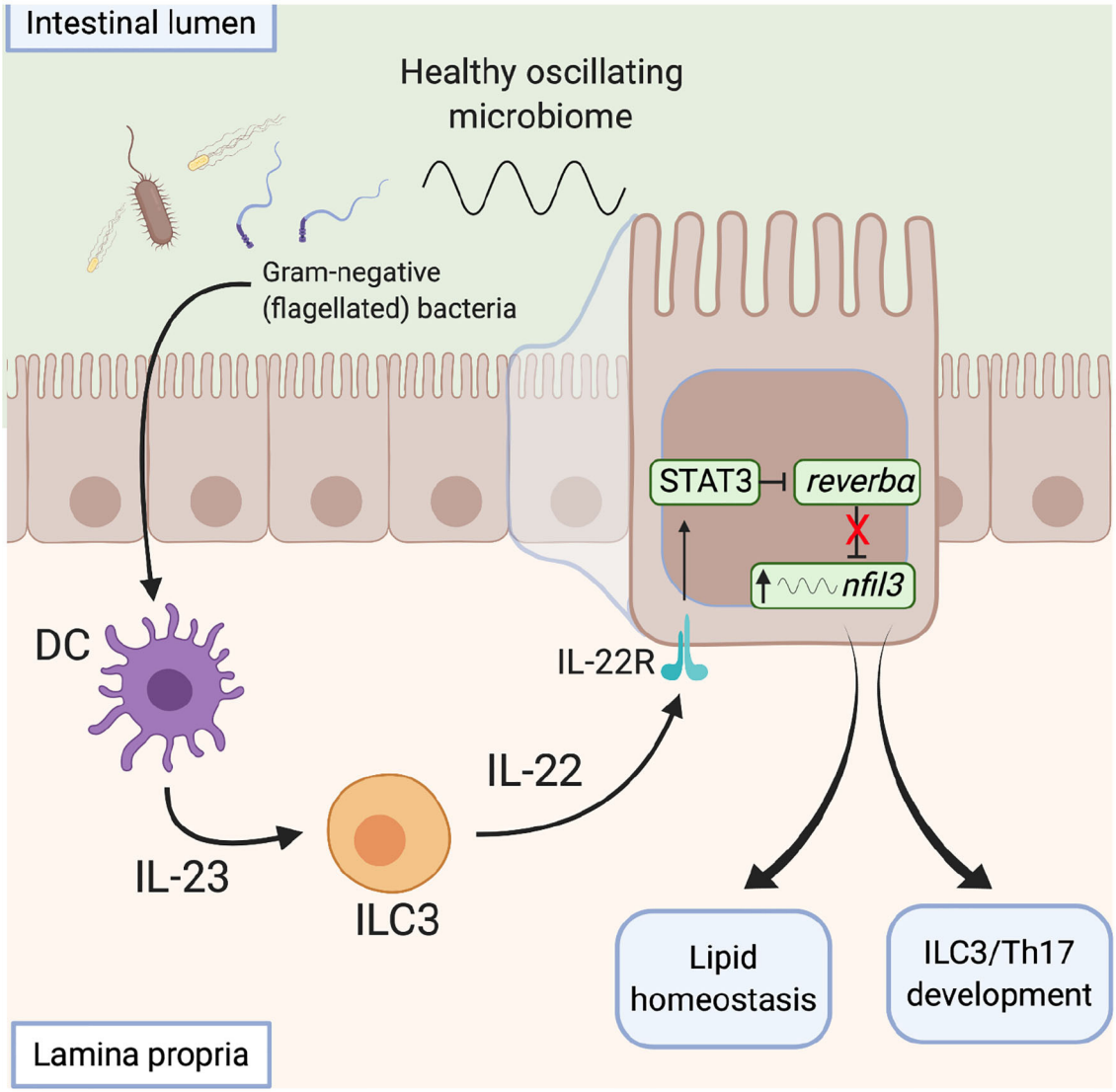

FIGURE 5 | The microbiota influences metabolic programmes within the gut. Signals derived from gram-negative flagellated bacteria resident within a healthy microbiome activate toll-like receptors on lamina propria CD11c^{+} dendritic cells and induce IL23 secretion. In response to increased IL23 levels, ILC3s signal to intestinal epithelial cells via IL22, which activates a STAT3 signaling pathway to repress *rev-erbα* expression, with subsequent de-repression of *nfil3* transcription. Increased *nfil3* expression drives circadian lipid homeostasis and development of ILCs and T_h17 cells, promoting intestinal homeostasis. DC, dendritic cell. ILC, innate lymphoid cell, STAT, signal transducer and activator of transcription.

transcriptional programmes (90). These studies provide proof-of concept that systemic metabolic rhythms are reliant on the rhythmic microbial outputs. It will be interesting to explore the extent to which these rhythmic signals direct other physiological processes, with immune homeostasis being an obvious candidate.

CONCLUSIONS AND FUTURE DIRECTION

The intestinal microbiome is very well established as an influential component of immune-metabolic homeostasis. A healthy microbiome exhibits rhythmicity in microbial composition, as well as rhythmicity in its biophysical location and metabolic outputs, each of which will influence immune homeostasis. Whilst evidence is emerging of gut commensals with potential intrinsic rhythmic capacity, it is likely that these microbiome rhythms will be heavily reliant on entrainment derived largely from the host. This may take the form of food timing, food composition, secreted host signals or non-dietary intake including antibiotics and immunomodulatory medication, as well as host disease state. Initial observations of microbiome rhythmicity in immune homeostasis and associated dysrhythmia in inflammatory disease states should drive scientific investigation into the mechanisms of host circadian

regulation of the microbiome and *vice versa*, as it is not yet clear which immune components are directly sensitive to changes in microbiome rhythmicity and which host-microbiome pathways predominate in health and disease. Whilst current studies have predominantly focused on the metabolic implications of an arrhythmic microbiome, future work should expand on early work exploring the impact of microbiome rhythmicity on composition and function of immune subsets. Piecing together the relative contribution of each moving part in the microbiome, intestinal epithelium and circulating immune cells will help to understand the role of circadian rhythmicity in crosstalk between the microbiome and immune system.

Evidence of microbiome dysbiosis associated with chronic human inflammatory conditions is growing, however, addition of a circadian component to human immuno-microbiome trials is in its infancy. In humans, manipulation of the microbiome via fecal microbial transplantation is already established to modulate immune response in infective conditions such as *Clostridium difficile* infection and is under investigation in inflammatory conditions such as inflammatory bowel disease (128, 129). It is exciting to consider circadian influence on microbiome health as a pathway to further understanding of mucosal immunology and ultimately improve efficacy of microbiome modulation as a chronotherapy for human inflammatory and auto-immune disease.

AUTHOR'S NOTE

Figures were created with Biorender.com.

AUTHOR CONTRIBUTIONS

TB and JG wrote the manuscript and designed the figures. All authors contributed to the article and approved the submitted version.

FUNDING

Work in JG's laboratory is funded by the MRC (MR/S002715/1 and MR/P023576/1). TB is an MRC Clinical Research Training Fellow (MR/S02199X/1).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors JG.

frontiers in Immunology

REVIEW
published: 10 September 2020
doi: 10.3389/fimmu.2020.552188

Diurnal Rhythmicity Programs of Microbiota and Transcriptional Oscillation of Circadian Regulator, NFIL3

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Circadian rhythms are a very exquisite mechanism to influence on transcriptional levels and physiological activities of various molecules that affect cell metabolic pathways. Long-term alteration of circadian rhythms increases the risk of cardiovascular diseases, hypertension, hypertriglyceridemia, and metabolic syndrome. A drastic change in dietary patterns can affect synchronizing the circadian clock within the metabolic system. Therefore, the interaction between the host and the bacterial community colonizing the mammalian gastrointestinal tract has a great impact on the circadian clock in diurnal programs. Here, we propose that the microbiota regulates body composition through the transcriptional oscillation of circadian regulators. The transcriptional regulator, NFIL3 (also called E4BP4) is a good example. Compositional change of the commensal bacteria influences the rhythmic expression of NFIL3 in the epithelium, which subsequently controls obesity and insulin resistance. Therefore, control of circadian regulators would be a promising therapeutic target for metabolic diseases.

Keywords: circadian rhythms, microbiota, metabolic diseases, cytokine, obesity

OPEN ACCESS

Edited by:
Christoph Scheiermann, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Charna Dibner, Université de Genève, Switzerland
Mihaela Gadjeva, Harvard Medical School, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *15 April 2020*
Accepted: *18 August 2020*
Published: *10 September 2020*

Citation:
Kubo M (2020) Diurnal Rhythmicity Programs of Microbiota and Transcriptional Oscillation of Circadian Regulator, NFIL3. Front. Immunol. 11:552188. doi: 10.3389/fimmu.2020.552188

INTRODUCTION

Obesity is a major risk factor for several co-occurring diseases, including type II diabetes mellitus, non-alcoholic fatty liver disease, and ischemic cardiovascular disease, and the prevalence of these diseases has increased at an astounding rate in the past decades (1). About 44% of the global population is overweight, and more than 300 million individuals are affected by morbid obesity (2). This is thought to be the result of dramatic changes in the human lifestyle, ranging from a drastic change in dietary patterns, improved hygiene, and altered sleep cycles. Therefore, there is an urgent need to identify host and environmental factors that regulate human metabolism and energy homeostasis. In considering these two aspects, the intestinal flora is an environmental factor that greatly affects the body composition of mammals (3). The gut flora facilitates energy

Abbreviations: BMAL1, brain and muscle ARNT-like 1; cDC1, conventional dendritic cell 1; CLOCK, circadian locomotor output cycles kaput; CLPs, common lymphoid progenitors; CRY, Cryptochrome; IBD, inflammatory bowel disease; ILCs, innate lymphoid cells; LD cycle, light-dark cycle; LPS, lipopolysaccharide; MYD88, Myeloid differentiation primary response 88; PER, period; ROR, retinoic acid receptor-related orphan receptor; SCN, suprachiasmatic nucleus; TLR9, toll-like receptor 9; ZT4, Zeitgeber time 4.

collection when energy derived from the diet is stored in adipose tissue (4). Recently, there is some evidence indicating a role for temporal and spatial dynamics in the community of microorganisms that inhabit the gastrointestinal tract. The circadian clock evolved in most species to adjust the physiology of the organism to daily environmental fluctuations (5–7). Epidemiological and experimental evidence has demonstrated that clock disturbances are linked to metabolic diseases, including obesity and hyperglycemia (8, 9). In understanding how regulation of microbial host metabolic pathways affects energy storage and body composition, we propose that the microbiota regulates body composition through the clock regulating transcription factor NFIL3 (also called E4BP4), which influences the circadian clock in intestinal epithelial cells through the regulation of group 3 innate lymphocyte cells (ILC3). This review describes how NFIL3 regulates body composition and establishes an essential network between the circadian clock and host metabolism.

CIRCADIAN CLOCK AND METABOLIC DISORDERS

Circadian rhythms are a very exquisite mechanism by which organisms can adapt their behaviors to the 24-h light-dark (LD) cycle change in the external environment evoked by the rotation of the earth around the sun (10). Transcriptional levels and physiological activities of various molecules that affect cell metabolic pathways and organ functions have their own periodicity, which is known to be very consistent with the LD cycle of the external environment. Synchronizing the circadian clock with the metabolic system is necessary to make dietary substrates available for metabolic pathways that are energetically expensive and plays an important role in optimizing energy use. The intrinsic circadian clock is entrained by LD cycles, and the mammalian master clock resides in the suprachiasmatic nucleus (SCN), a small area of the anterior hypothalamus. The clock plays a role to drive oscillators distributed in various peripheral tissues through behavioral and neuroendocrine signals (11). Peripheral tissues have functional clock oscillators that are self-sustained and can operate independently of the central pacemaker and SCN rhythms (12). For instance, the temporal pattern of food intake influences the quality and quantity of the circadian transcriptome in the mouse liver. Therefore, while the LD cycle resets the master clock in the SCN, the timing of food intake can be a potent synchronizer of peripheral clocks.

Disruption of the circadian clock due to a genetic defect has been shown to have a strong causal relationship with metabolic diseases (13, 14). Indeed, sleep restriction or a 28-h LD cycle reduces insulin sensitivity and glucose tolerance (15). This has been demonstrated in epidemiological studies indicating that long-term alteration of sleep patterns increases the risk of obesity and metabolic disorders (16). Furthermore, the prevalence of cardiovascular diseases, hypertension, hypertriglyceridemia, and metabolic syndrome is higher in shift workers compared to day-time workers, and restriction of sleep time has been shown to increase the risk of obesity and diabetes. In this context, the temporal and spatial dynamics of the microbial flora may have a profound effect on host metabolism by tightly associating with the circadian clock.

A layer of intestinal epithelial cells (IECs) provides the physical barrier that separates commensal bacteria living in the intestinal lumen from the body. Interactions between commensal bacteria and Toll-like receptors (TLRs) members of the pattern recognition receptors (PRRs) are known to be mandatory for IEC homeostasis maintained by host-commensal symbiosis. Several functions in IECs, including nutrient absorption, cell proliferation, motility, and metabolic activities are regulated in a circadian rhythm. The circadian variation in the host intestine is tightly associated with diurnal oscillations of the intestinal microbiota colonizing the mammalian gastrointestinal tract, thus the oscillations of the circadian clock in IELs are controlled by the timing of food intake and the composition of the diet, which affects the expression pattern of some TLR genes. Moreover, the functional feature generated by the microbiota oscillations feeds into the circadian clock network not only in the intestine but also in the system as a whole (17).

Recent evidence indicates that alterations in the composition of the microbiome change the susceptibility to obesity of the host (18). Obesity and diabetes are known to cause chronic hyperglycemia in IELs, leading to a breakdown of barrier function that facilitated the alteration of intestinal bacterial components (19). Therefore, a chronic increase of glucose levels in obesity contributes to a detrimental effect in the maintenance of a barrier function in the IELs as a consequence of the functional reprogramming of metabolism and transcription. Subsequently, the perturbation in the coordinated daily interplay between the microbiota oscillations and the circadian transcriptional program in IELs enhances the risk for the development of obesity and systemic inflammation.

The composition and function of the bacterial community colonizing the mammalian gastrointestinal tract also undergo oscillations, which are affected by the dietary condition (14, 20, 21). The interaction between the host and gut microbiome can affect the circadian clock in different tissues (22, 23). Systemic metabolite rhythms and programming of transcriptional oscillations impact the homeostatic diurnal variation in the liver (22). These observations indicate that the microbiome is a major source of clock-modifying metabolites.

MOLECULAR ARCHITECTURE OF THE CIRCADIAN CLOCK IN THE GUT

Circadian rhythm is controlled by a core loop composed of the heterodimeric complex of two transcriptional activators, the circadian locomotor output cycles kaput (CLOCK), and brain and muscle ARNT-like 1 (BMAL1) (**Figure 1**) (24). The CLOCK and BMAL1 form the heterodimer via their HLH-PAS domains, and the heterodimer of transcriptional activators subsequently results in translocation to the nucleus, where it binds to an E-box sequence (CACGTG) in the promoter regions of repressors of CLOCK/BMAL1-mediated transcription, such as Period (PER 1 to 3) and Cryptochrome (CRY 1 and 2) genes. CRY inhibits

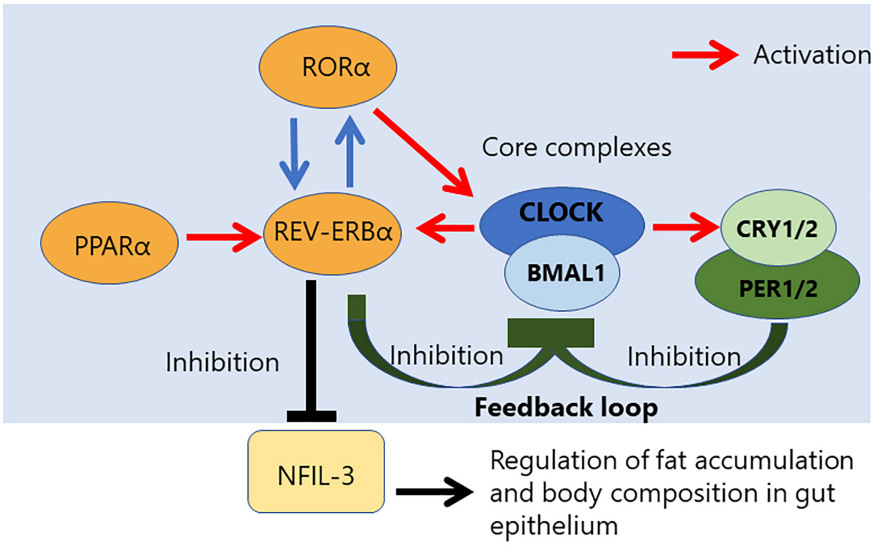

FIGURE 1 | Relationship between NFIL-3 and the core complex controlling the circadian clock. Circadian rhythm is regulated by a transcriptional feedback loop composed of three core complexes. CLOCK/NPAS2/BMAL1 complex. NFIL-3 is a part of accessory loop, which regulates fat accumulation and body composition in gut epithelium.

the histone acetyltransferase p300, leading to a decrease of CLOCK/BMAL1-mediated transcription (25). The stability of the PER and CRY proteins is regulated by specific E3 ubiquitin ligase complexes, and the CLOCK/BMAL1 vs. CRY/PER mutually regulates each other via the central autoregulatory feedback loop (**Figure 1**). Therefore, this feedback loop is important to determine the periodicity of the circadian oscillation (26, 27).

Additional transcriptional feedback loops that are composed of members of the retinoic acid-related orphan nuclear receptor family, REV-ERBα/β repressor and RORα activator, ensure the stability and fidelity of the molecular clock (28). The antiphasic expression of the RORα and RevErbα controls a rhythmic expression of TLR in IELs (22). REV-ERB and its antagonist receptor RORα also competitively bind to the promoter region of the *Bmal1* gene to control rhythmic chromatin dynamics (29–32). The transcription regulator of lipid metabolism, peroxisome proliferator-activated receptor a (PPARα) is a critical activator of RevErbα expression (22). Activation of PPARα is known to promote many aspects of fatty acid metabolism. On the other hand, BMAL1 controls the rhythmic expression of short-chain fatty acids (SCFA) *receptor, Ffar2/3,* in the colonic muscle layer. Moreover, some evidence is indicating that diurnal microbial SCFA levels also influence on intestinal motility (33, 34).

The circadian rhythm associated genes directly contribute to the pathogenesis of intestinal diseases. Sleep disruption and chronic fatigue are the major complaints of patients with inflammatory bowel disease (IBD), and these symptoms affect the inflammatory process of the disease (35–37). The loss of BMAL1 disrupts both the circadian clock and the timing of regeneration in the mouse intestine controlled by TNF (38). Deficiency of Per1/2 results in not only decreased proliferation of intestinal stem cells (39) but also increased cell death of intestinal epithelial cells in the lower hemicrypts (40). In humans, a polymorphism in Per3 has been associated with increased susceptibility to and disease severity of IBD (41). Moreover, deficiency of Per1/2 controlled Wee1 plays a role in mitotic cell cycle arrest, resulting in increased susceptibility of the gut epithelium to inflammatory processes (42).

In the intestinal immune response, it has been reported that an essential role of gut-resident macrophages, particularly residents in lamina propria, contributes to host defense (43). The transcriptional profile in self-maintaining macrophage has a great impact on their localization in peripheral tissue. Therefore, impairment in diurnal rhythmicity programs of transcription may result in a reduction of intestinal functions.

ROLE OF THE CLOCK REGULATOR NFIL3 AND THE CIRCADIAN CLOCK IN THE IMMUNE SYSTEM

NFIL3 was originally identified as a transcriptional repressor that binds to the E-box that controls the circadian clock (44); it is located in an auxiliary loop that exists outside the core loop (45). NFIL3 is a basic leucine zipper transcriptional factor that is mainly expressed in DC, T cells, and various other immune cells. This factor is required for the differentiation of the conventional dendritic cell 1 (cDC1) subset involved in cross-presentation (46, 47). Recently, NFIL3 expression was found to be required for the transition stage of cDC1 progenitors through the *Nfil3–Zeb2–Id2* pathway that controls the Irf8 enhancer switch (48). NFIL3 is expressed in common lymphoid progenitors (CLPs) and regulates the expression of Id2 and Eomes genes that are important for NK cell development (49, 50). Indeed, NFIL3 deficiency causes an intrinsic defect in NK cell development. NFIL3 also controls the differentiation of several other innate

lymphoid cells (ILCs), including group 2 ILC (ILC2) and ILC3 cells, through the repression of Id2 in CLPs (51, 52). NFIL3 is a common regulator directing the development of CLPs that differentiate into all ILC lineages (53).

Regulation by circadian clocks has been described in the innate immune system because the CLOCK/BMAL1 complex regulates the expression of Toll-like receptor 9 (TLR9) and represses the expression of REV-ERBα, suppressing the induction of interleukin-6 (54). In contrast, the role of the circadian clock in the adaptive immune system seems to be controversial. Many previous studies indicated that the cell-intrinsic circadian clock is involved in different aspects of adaptive immune function. But, mice with a T-cell-specific deletion of Bmal1 had normal differentiation of T_H17 cells (55), suggesting no intrinsic role of circadian clocks in the T cell response of the intestinal tract. On the other hand, NFIL3 was suggested to act as a repressor of a key driver of nuclear receptor RORgt, which is an essential intrinsic transcriptional factor for T_H17 cell differentiation. Therefore, the diurnal expression of NFIL3 is regulated by the circadian network through direct repression of REV-ERBa, which binds to the consensus sequence of the *Nfil3* locus and represses NFIL3 expression (**Figure 1**). Therefore, NFIL3 expression in T cells plays a role in controlling the intestinal immune response regulated by T_H17 cells (45). For T_H1 cell immune function, NFIL3 has a different role to induce IL-10 and IL-13 expression (56) and, in this case, the expression of NFIL3 is induced by excessive IFN-γ stimulation to induce IL-10 and IL-13.

TRANSCRIPTIONAL OSCILLATION OF NFIL3 IN INTESTINAL EPITHELIAL CELLS

The defense at barrier surfaces by the gut epithelium plays a role in the containment of commensal bacteria. The barrier function of the gut epithelium is regulated by $CD4^+$ T cell responses against commensal bacteria (57) and by antibacterial proteins derived from gut epithelial cells (58). NFIL3 is expressed by the small intestine epithelium under an LD cycle change, and this circadian expression is significantly altered in antibiotic-treated or germ-free mice (33). The expression of NFIL3 in the epithelium influences the response to commensal bacteria. Interestingly, intestinal epithelial cell-specific deficiency of NFIL3 has a great impact on the regulation of lipid storage and epididymal fat weight. Therefore, the expression of NFIL3 in the gut epithelium plays an important role in the regulation of lipid storage and body composition.

NFIL3 expression is known to be regulated by the core circadian clock transcriptional repressor REV-ERBα which binds to the *Nfil3* locus, resulting in circadian expression of NFIL3 (45, 59). Intestinal epithelial cells sense commensal bacteria via TLRs and the signaling adapter molecule, Myeloid differentiation primary response 88 (MyD88), and these innate signaling pathways promote NFIL3 expression via suppression of REV-ERBα expression in the epithelium. Interestingly, the expression pattern of REV-ERBα and NFIL3 in Zeitgeber time 4 (ZT4) are mutually exclusive in epithelial cells.

The DC-ILC3 network can be activated by flagellin or lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria (60, 61). The penetration of gram-negative bacteria into the intestinal epithelial barrier allows them to contact lamina propria DC and activates $CD11c^+$ DC to produce interleukin 23 (IL-23). This process subsequently leads to further activation of group 3 innate lymphoid cells (ILC3s). ILC3s are reported to play an important role in defense at intestinal barrier surfaces via IL-17 and IL-22 production (62). The DC-derived IL-23 promotes IL-22 production by ILC3s, subsequently leading to the up-regulation of NFIL3 in the intestinal epithelium (63). In this case, NFIL3 plays a role to regulate lipid absorption and export in intestinal epithelial cells via promoting the expression of several molecules controlling lipid metabolism, including a member of the class B scavenger receptor family, CD36, which is a transporter of dietary fatty acids, stearoyl-coenzyme A-desaturase 1 (SCD1), a fatty acid hydroxylase, CYP2E1, and a fatty acid-binding protein 4 (FABP4) (64–67). This finding is consistent with the observation in a loss of function mutant of NFIL3 that lipid metabolism is partially altered (68).

Therefore, network regulation of the microbiota and the circadian clock in the intestinal tract is a critical process to control obesity and insulin resistance (18). Transcriptional oscillation of circadian regulators like NFIL3, which are controlled by the diurnal rhythmicity of the microbiota, is an important program for host metabolism.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 18 September 2020
doi: 10.3389/fimmu.2020.02143

Glucocorticoids Regulate Circadian Rhythm of Innate and Adaptive Immunity

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Animals have evolved circadian rhythms to adapt to the 24-h day-night cycle. Circadian rhythms are controlled by molecular clocks in the brain and periphery, which is driven by clock genes. The circadian rhythm is propagated from the brain to the periphery by nerves and hormones. Glucocorticoids (GCs) are a class of steroid hormones produced by the adrenal cortex under the control of the circadian rhythm and the stress. GCs have both positive and negative effects on the immune system. Indeed, they are well known for their strong anti-inflammatory and immunosuppressive effects. Endogenous GCs inhibit the expression of inflammatory cytokines and chemokines at the active phase of mice, regulating the circadian rhythm of tissue inflammation. In addition, GCs induce the rhythmic expression of IL-7R and CXCR4 on T cells, which supports T cell maintenance and homing to lymphoid tissues. Clock genes and adrenergic neural activity control the T cell migration and immune response. Taken together, circadian factors shape the diurnal oscillation of innate and adaptive immunity. Among them, GCs participate in the circadian rhythm of innate and adaptive immunity by positive and negative effects.

Keywords: circadian rhythm, glucocorticoid, IL-7 receptor, inflammatory cytokine, T cell

OPEN ACCESS

Edited by:
Gustavo Javier Martinez, Rosalind Franklin University of Medicine and Science, United States

Reviewed by:
Laura A. Solt, The Scripps Research Institute, United States
Nicolas Cermakian, McGill University, Canada

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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *26 March 2020*
Accepted: *06 August 2020*
Published: *18 September 2020*

Citation:
Shimba A and Ikuta K (2020) Glucocorticoids Regulate Circadian Rhythm of Innate and Adaptive Immunity. Front. Immunol. 11:2143. doi: 10.3389/fimmu.2020.02143

INTRODUCTION

Circadian rhythms are endogenously regulated by molecular clocks, which can be controlled by the 24 h day-night cycle. Light and dark stimuli are converted into neuronal activity in the retina and transmitted to the suprachiasmatic nucleus (SCN) of the brain. This neuronal activity induces neuropeptide production in the brain, which stimulates the synthesis of hormones in endocrine organs. Together with sympathetic neurons, the hormones integrate circadian rhythms in peripheral organs. The circadian rhythm controls physiological activities in the body, such as metabolism in liver, blood pressure, sleep, and immune response. Especially, immune functions such as cytokine expression, leukocyte mobilization, and antigen priming show diurnal changes. Past studies have revealed that clock genes, nervous signals, and hormones control the circadian rhythm of the immune system. Especially, glucocorticoids (GCs), a group of steroid hormones produced by the adrenal cortex, affect the circadian rhythm of the immune system. In this article, we will first explain the general functions of GCs in the circadian control of the immune system,

then introduce our recent works, and finally discuss potential interactions between GCs and other circadian mediators in immune regulation.

GLUCOCORTICOIDS AND CIRCADIAN RHYTHMS

At steady state, GC production is under the control of circadian rhythms (1). Blood GC levels oscillate with a peak in early morning and nadir at night in diurnal animals like humans and in reverse fashion in nocturnal animals like rodents. Neuronal activity in the SCN induces the paraventricular nucleus (PVN) of the hypothalamus to secrete corticotropin-releasing hormone (CRH). CRH then stimulates the anterior pituitary, leading to the production of adrenocorticotropic hormone (ACTH) in the blood. ACTH finally stimulates the adrenal cortex to produce GCs. In addition, GCs are also induced by endogenous and physiological stress (1).

Glucocorticoids exert their functions through binding to intracellular GC receptor (GR) (2). GR consists of an N-terminal activation function-1 (AF-1) domain, DNA-binding domain (DBD), and ligand-binding domain. GC-GR binding induces GR to dimerize, translocate to the nucleus, and bind to specific DNA sequences, known as GC-response elements (GREs), to activate or repress the transcription of target genes. Based on the gene response, GREs are classified as positive or negative (nGREs). Because mineralocorticoid receptor (MR) and GR are related steroid hormone receptor and share their basic structure of nuclear receptor, GCs can bind to both of GR and MR, suggesting that endogenous GCs work through GR and MR to regulate immunity (3). In addition, GR directly interacts with other transcription factors, such as NF-κB, AP-1, MAPK, and STAT, to regulate their functions.

The molecular clock controls circadian rhythm by transcription-translation feedback loop of clock genes (4, 5). The core clock components, BMAL1 and CLOCK, form a heterodimeric complex that stimulates the transcription of E-box containing genes of other molecular clock components, including PERs, CRYs, REV-ERBs, and RORs. As the core clock loop, the negative feedback in which PER/CRY complex repress their own transcription by interacting with BMAL1/CLOCK complex drives the 24-h oscillation of PER/CRY expression. Additionally, REV-ERBs and RORs are parts of the accessory loop that represses or induces the expression of BMAL1 and NFIL3. NFIL3, as the downstream mediator of the molecular clock, induces the expression of PERs, REV-ERBs, and RORs. GCs also regulate the expression of some clock components such as PER1, PER2, REV-ERBα, and NFIL3 (6–8). Especially, GRE motifs are present in *Per1, Per2,* and *Nfil3* gene loci (6). So, central nervous system directs the diurnal production of GCs, and GCs have function to integrate the circadian rhythm of peripheral organs.

The diurnal cycle of GCs also modulates metabolism. Because GR directly induces the expression of metabolic enzymes such as phosphoenolpyruvate carboxylase 1 (PCK1) and glucose-6-phosphatase catalytic subunit (G6PC), GCs enhance gluconeogenesis in the liver and lipolysis in adipose tissues to release fatty acid and glycerol (9). Moreover, GCs can enhance the effects of adrenergic neural activity, known as permissive effects. For example, GCs increase the sensitivity of skeletal muscle to adrenaline (10). GCs also enhance glucagon-, adrenaline-, and cyclic AMP-mediated gluconeogenesis; these metabolic pathways are attenuated in adrenalectomized mice (9). Therefore, the circadian oscillation of GCs induces a diurnal change of metabolic activity in peripheral organs.

IMMUNOSUPPRESSIVE EFFECTS

Glucocorticoids help regulate the circadian expression of a number of inflammatory cytokines. GCs are well known for their strong anti-inflammatory and immunosuppressive effects and are thereby widely used for treatment of inflammatory and autoimmune diseases (11, 12). First, GR carries out immunosuppressive roles by activating or suppressing gene expressions via binding to GREs. For example, GR transactivates anti-inflammatory factors, GILZ and IκBα, whereas GR suppresses the expressions of inflammatory cytokines and factors such as IL-6 and C3 through nGRE (13). Second, GR can suppress the inflammatory factor expression by interacting directly with other transcriptional factors such as NF-κB and AP-1 as transrepression. GR interacts with DNA-bound NF-κB and AP-1 and suppresses their activities by recruiting the transcription repressor such as nuclear receptor corepressor and by inhibiting the recruitment of transcriptional coactivators such as nuclear coactivator and the detachment from DNA (14, 15). In addition, recent studies reported other mechanisms that GR regulates the transcription of AP-1 via GR-binding to half-site of cryptic GRE embedded in AP-1 response elements (TREs) (16), and DBD of GR can directly bind in a DNA-dependent manner to NF-κB response elements (17). By these mechanisms, GCs inhibit the synthesis of various cytokines including IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, IL-18, GM-CSF, TNF-α, and IFN-γ (18–21).

Researchers have also showed *in vivo* effects of GCs to suppress immune responses in rodents. In a mouse model of inflammation, LPS-stimulated GR-deficient macrophages show a higher expression of several inflammation-associated genes such as IL-6, TNF-α, and COX-2, leading to lethality (22). This effect is because GR directly associates with p38 MAPK and inhibits the latter's activity through MAPK phosphatase-1 induction. Like macrophages, GCs also suppress the responses of dendritic cells (DCs). DC-specific (CD11c-Cre) GR-deficient mice produce higher amounts of the inflammatory cytokines IL-1β, TNF-α, and IL-12 (23). In lymphocytes, GCs suppress Th1 cell differentiation and reduce IFN-γ production by Th1, CD8 T, and NK cells, leading to the inhibition of cytotoxic responses (24–26). The deficiency of GR causes the increased production of IFN-γ from Th1 and NK cells to lead the higher mortality in the infection of toxoplasma and mouse cytomegalovirus (27–29).

Glucocorticoids are partly involved in regulation of the circadian rhythm of immune responses by clock genes. The expression of inflammatory cytokines by innate immune cells

after LPS stimulation exhibits circadian oscillation due to diurnal change of the expression of TLR and clock genes (5). Wang et al., reported that circadian oscillation of response to endotoxin was disrupted in *Per2* mutant (Per2m) mice and that the Per2m mice showed alleviated endotoxin shock (30, 31). Interestingly, corticosterone concentration became higher in Per2m mice after LPS administration, and the increased corticosterone might have suppressed the inflammation and mortality of Per2m mice. They showed that the upregulation of CLOCK and BMAL1 due to *Per2* mutation induced the expression of steroidogenic acute regulatory protein (StAR), a rate-limiting enzyme in GC synthesis, by binding to the promoter of *Star* gene. Thus, the molecular clock can control the circadian rhythm of inflammation by regulating GCs synthesis.

Glucocorticoids also control the circadian rhythm of chemokine expression. Gibbs et al., found that CXCL5, a chemokine recruiting neutrophils and monocytes at the inflammatory site, is produced in mice in a diurnal fashion under the control of diurnal GC secretion (32). CXCL5 expression is suppressed through GR binding to nGREs in the CXCL5 promoter. In mice with lung inflammation induced by LPS, CXCL5 expression shows a diurnal oscillation with a peak at daytime, triggering the diurnal accumulation of neutrophils in the lung. The levels of inflammatory cytokines such as IL-6, TNF-α, and G-CSF increase shortly after the peak of CXCL5 and neutrophil count. Because GCs shows an anti-phase diurnal oscillation with CXCL5 and neutrophils, neutrophil migration might be reduced at night via the repression of CXCL5 expression by GCs. In fact, the Gibbs study further reported that the circadian change of CXCL5 and neutrophils is impaired in adrenalectomized mice. BMAL1 is also involved with circadian rhythm of inflammation via macrophages and neutrophils (33). Although a circadian change of CXCL5 was not affected in airway club cell-specific (CCSP-Cre) BMAL1-deficient mice, the diurnal change of neutrophil infiltration into lung was impaired, and inflammation was enhanced, suggesting that BMAL1 in club cells plays some role in controlling circadian rhythm of neutrophil migration and immunosuppression independently of CXCL5 (32). The study also found that the daytime increase of neutrophils efficiently protects mice against bacterial infection by *S. pneumoniae*. However, the same group reported later that the airway club cell-specific deletion of GR caused a normal circadian rhythm of LPS-mediated neutrophil infiltration despite the loss of CXCL5 circadian rhythm (34). Additionally, macrophage-specific (LysM-Cre) deletion of GR resulted in the loss of the circadian oscillation of CXCL5 but not of neutrophils. These reports indicate that GCs regulate the circadian rhythm of CXCL5 expression in lung club cells and myeloid cells, but that neutrophil infiltration is not regulated by GCs. Nevertheless, there are mechanisms of neutrophil migration in the lungs that still need to be worked out.

IMMUNE-ENHANCING EFFECTS

In addition to their negative effects, GCs can have positive effects on the immune system. Wiegers et al., reported that cytokine receptors for IL-2, IL-6, IFN-γ, GM-CSF, and TNF-α were potently upregulated in various types of cells by GCs (35), and Franchimont et al., reported that dexamethasone (DEX) treatment enhanced the expression of IL-7 receptor α-chain (IL-7Rα) in human blood T cells (36). IL-7 is a cytokine essential for the development and maintenance of lymphocytes (37–39). IL-7R consists of the IL-7Rα chain and common γ-chain, and induces the phosphorylation of STAT5 and PI3 kinase. This signal transduction promotes the survival and proliferation of T cells, B cells, and innate lymphoid cells (ILCs), and supports their maintenance in peripheral lymphoid organs. Furthermore, IL-7 rescues T cells from apoptosis induced by GCs, and IL-7Rα upregulated by DEX enhances IL-2Rα expression and IL-2–induced survival (36).

To investigate the mechanism how GCs regulate IL-7Rα expression, Lee et al., searched the IL-7Rα locus and found DNA sequences conserved among different species at 3.6 kb upstream of the promoter (40). They designated this region non-coding conserved sequence 1 (CNS-1). CNS-1 is about 300 bp long and has 86% homology between human and mouse. It also has two GRE motifs and one NF-κB motif that are conserved. Point mutations in GREs impair transactivation of the IL-7Rα promoter by GCs. Later, Abe et al., generated IL-7Rα CNS-1 deletion mice and tested the *in vivo* role of CNS-1 in IL-7Rα expression (41). Although IL-7Rα expression was unchanged in thymocytes, CNS-1-deletion mice exhibited a reduction of IL-7Rα expression in peripheral T cells, the absolute numbers of which were decreased in spleen and lymph nodes. Moreover, IL-7Rα induction by GCs was lost in T cells of CNS-1 deletion mice. Thus, CNS-1 is a proximal enhancer of the IL-7Rα locus regulated by GCs.

To clarify the biological significance of IL-7Rα induction by GCs, we generated the mice harboring point mutations in two GREs of the CNS-1 (GRE point mutant mice, GREm mice) and analyzed them together with T cell-specific (CD4-Cre) GR-deficient mice (42). IL-7Rα expression on T cells is elevated at night and diminished at daytime in control mice, which is consistent with blood GC levels. Human blood T cells also show a diurnal change (43). Of interest, mouse T cells accumulate in secondary lymphoid organs, such as spleen, lymph nodes, and Peyer's patches at night. By contrast, blood T cells accumulate at day. These results suggest that T cell recirculation between secondary lymphoid organs and blood has a diurnal oscillation. This diurnal oscillation is lost in T cell-specific GR-deficient and GREm mice. GC rhythms have also been linked to the diurnal change of T cell blood counts in humans. It was shown that cortisol induces chemokine receptor CXCR4 and that CXCR4 expression was reduced by mifepristone, an antagonist of steroid hormone receptors including GR, or by metyrapone, an inhibitor of GC synthesis, impairing the diurnal oscillation of T cells in human blood (44, 45). The observation supports the idea that the involvement of CXCR4 as a mediator for the effects of GCs on T cells is conserved both in humans and rodents. In addition, Besedovsky et al., showed that administration of spironolactone, an antagonist for mineralocorticoid receptor (MR), increased T cell counts in human peripheral blood at night. Because MR has the potential to bind to GCs, it suggests that GCs at low

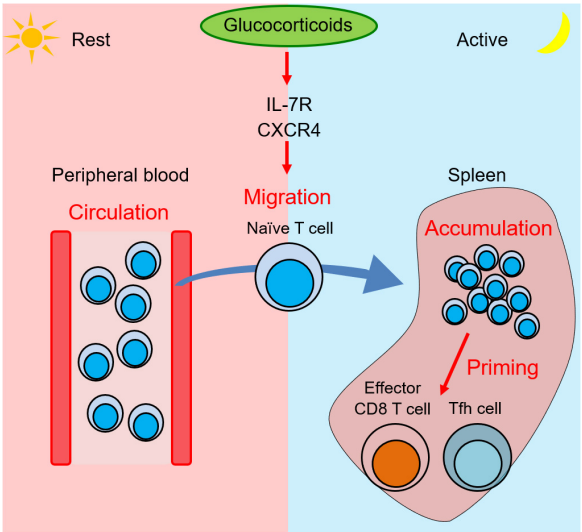

FIGURE 1 | Glucocorticoids induce T cell migration into spleen from blood and enhance immune response at night by expression of IL-7R and CXCR4. Zeitgeber time (ZT) is a unit of cycle consisted of 12 h light/12 h dark phase. ZT0 is defined as the time when light on and ZT12 is defined as the time when light off. At the rest phase (ZT4) in mice, more T cells circulate in peripheral blood. At the onset of the active phase, the adrenal cortex releases glucocorticoids, which induce IL-7R and CXCR4 expression on T cells. At the active phase (ZT16), the elevated receptors trigger T cell migration into second lymphoid tissues such as spleen, lymph nodes, and Peyer's patches. The T cell accumulation induces a stronger immune response by effector CD8 T cells and follicular helper T (Tfh) cells against bacterial infections and soluble antigens.

concentrations bind to MR and control the circadian rhythm of T cell distribution (46). CXCR4 expression on T cells shows a diurnal oscillation similar to IL-7Rα in control mice, but this pattern is abolished in T cell-specific GR-deficient and GREm mice. Additionally, the diurnal change of the T cell distribution is lost in T cell-specific CXCR4-deficient mice, indicating that IL-7Rα induced by GCs is responsible for the diurnal rhythm of the T cell distribution. IL-7 also has the potential to induce CXCR4 expression on T cells (47). These results suggest that the diurnal change of GCs triggers the diurnal distribution of T cells (**Figure 1**).

The change of T cell accumulation in lymphoid organs during course of the day might affect immune responses against foreign antigens. Such a diurnal change of immune responses is observed in innate and adaptive immunity. Our data showed that T cell accumulation in lymphoid organs induced by GCs at night enhances the efficiency of effector CD8 T cells against bacterial infection, and follicular helper T (Tfh) cells, germinal center B cells, and class-switched B cells against soluble antigens (**Figure 1**). Similarly, we also reported that T cell accumulation at night is observed in Peyer's patches, where GR enhances germinal center formation and immunoglobulin class switching. Because it was reported that immune responses against *Salmonella* infection in gut is strongly induced at night (48), our findings imply that T cell accumulation in Peyer's patches at night might contribute to bacteria removal in mice.

GC receptor also influences the differentiation and function of helper T cell subsets. Correlated with these points, Th1 cell differentiation *in vitro* is increased in T cells of T cell-specific GR-deficient mice. By contrast, it is reported that GR promotes Th2 cell differentiation (49). Furthermore, GCs have the potential to promote the production of Th2 cytokines. In culture, primed CD4 T cells pretreated with DEX produce higher levels of IL-4, IL-10, and IL-13 (50). We also reported that the Th2 differentiation of GR-deficient T cells is impaired in Th2-skewed culture. *Nfil3* is a clock gene that promotes the production of Th2 cytokines (51). The *Nfil3* gene locus contains a GRE motif (6), and its expression is reduced in GR-deficient T cells in the early phase of Th2 differentiation. Moreover, the expression of IL-4 in Tfh cells by immunization with a soluble antigen is reduced in T cell-specific GR-deficient mice (42). These mice show reduced levels of plasma IgG1 and IgG2b, which are regulated by IL-4. After priming, Th2 cells become memory Th2 cells and stay long in the periphery (52). Memory Th2 cells cause chronic allergies, such as atopy and asthma (53). GCs also drive the circadian rhythm of IL-7Rα expression in memory Th2 cells (42). On the other hand, the diurnal rhythm of IL-7Rα and the maintenance of memory Th2 cells are impaired in T cell-specific GR-deficient and GRE mutant mice (42). Because GCs induce IL-7Rα expression in memory Th2 cells and IL-7 is important for the homeostasis of memory T cells, IL-7R induction by GCs likely supports the survival of memory Th2 cells. Taken together, GCs promote the differentiation of naive T cells into Th2 cells and the maintenance of memory Th2 cells.

Past studies have revealed that pathogenic memory Th2 cells play an important role in allergic response in asthma (53). Allergic responses in lung and skin exhibit time-dependent manner of symptoms. Nakamura et al., showed that mast cell-mediated diurnal rhythm of cutaneous anaphylactic reaction is dependent on clock genes and adrenal gland, and such rhythm is ablated in Clock mutant, *Per2* mutant, and adrenalectomized mice (54–57). Interestingly, they found that GCs not only suppressed the inflammation but also modulated the rhythm of clock genes expression, affecting the diurnal cycle and responsiveness (56, 57). On the other hand, mutation of clock genes impaired the diurnal cycle of GC production and GC-mediated suppression of mast cell activation (54, 55). Thus, clock genes and GCs mutually influence each other, and control the diurnal allergic reaction. However, there are few studies on the relationship between clock genes and the function of Th2 cells. Because Th2 cells might have resistance to the suppressive effects of GCs, the role of GCs on Th2 cell function remain unclear. So, it is to be clarified whether diurnal oscillation of GCs and clock genes shapes the circadian rhythm of allergic response by promoting the maintenance and function of pathogenic Th2 cells.

RELATIONSHIP BETWEEN GCs AND OTHER CIRCADIAN FACTORS ON IMMUNE REGULATION

Because GCs enhance the homing and response of T cells, GCs might suppress innate immunity but enhance adaptive

immunity. Similar to GCs, adrenergic receptors and the clock component BMAL1 in T cells control diurnal changes of the T cell distribution in lymph nodes, lymph, and blood. Druzd et al., found that the CCR7 expression on T and B cells show circadian oscillation with the peak at the active phase, but that the circadian oscillation of S1PR1 expression shows its peak at the rest phase in mice (58). BMAL1 enhances the CCR7-mediated recruitment of T and B cells to the lymph nodes and S1PR1-mediated egress. This mechanism leads to the circadian oscillation of the autoimmune response in an EAE model, in which Th17 cell number and symptoms were elevated at the active phase. Suzuki et al., showed that the signal from the adrenergic receptor, β2AR, augments the responsiveness of chemokine receptors CXCR4 and CCR7, and retains T and B cells in lymph nodes (59). The β2AR signal also enhances the differentiation of Tfh cells and immunoglobulin production by activated B cells. These effects lead to diurnal changes of the lymphocyte distribution and immune response. Thus, these studies suggest that adaptive immune responses are under the control of circadian rhythms through GCs, adrenergic receptor, and BMAL1.

In addition to the T cell distribution, other mechanisms also control the circadian rhythm of immune response. First, antigen presentation is important for the circadian rhythm of T cell response. Silver et al., reported that the expression of TLR9, CD80, and CD86 is high at the active phase, and CpG-OVA stimulation at the active phase efficiently induced the proliferation and cytokine production of T cells (60). Additionally, Nguyen et al., described circadian rhythm of monocyte distribution that the count of monocytes in spleen fluctuated during the course of the day with a peak at zeitgeber time (ZT)8 (61). Immune response against *L. monocytogenes* was induced strongly at ZT8 compared to ZT0, and BMAL1 regulated the circadian changes of the distribution, cytokine production, and bacterial rejection by monocytes. Sengupta et al., showed that BMAL1 regulated the diurnal difference of inflammation against influenza A virus which is induced strongly at the active phase via the monocyte infiltration in lung (62). Next, Fortier et al., found that T cell proliferation after anti-CD3 antibody stimulation became higher at the late rest or the active phase (circadian time (CT) 10-14) in a circadian manner, probably because of oscillating ZAP-70 expression with peak at CT 8 (63). It implies that intrinsic responsiveness of T cells follows the circadian rhythm. Same group also showed the antigen-presentation by OVA-pulsed DCs *in vivo* is more efficient at the rest phase (CT6) compared to at the active phase (CT18) (64). The circadian rhythm of T cell response persists even when using Bmal1-deficient DCs, whereas T cell-specific BMAL1-deficient mice lose the rhythm completely. Therefore, DC rhythms may

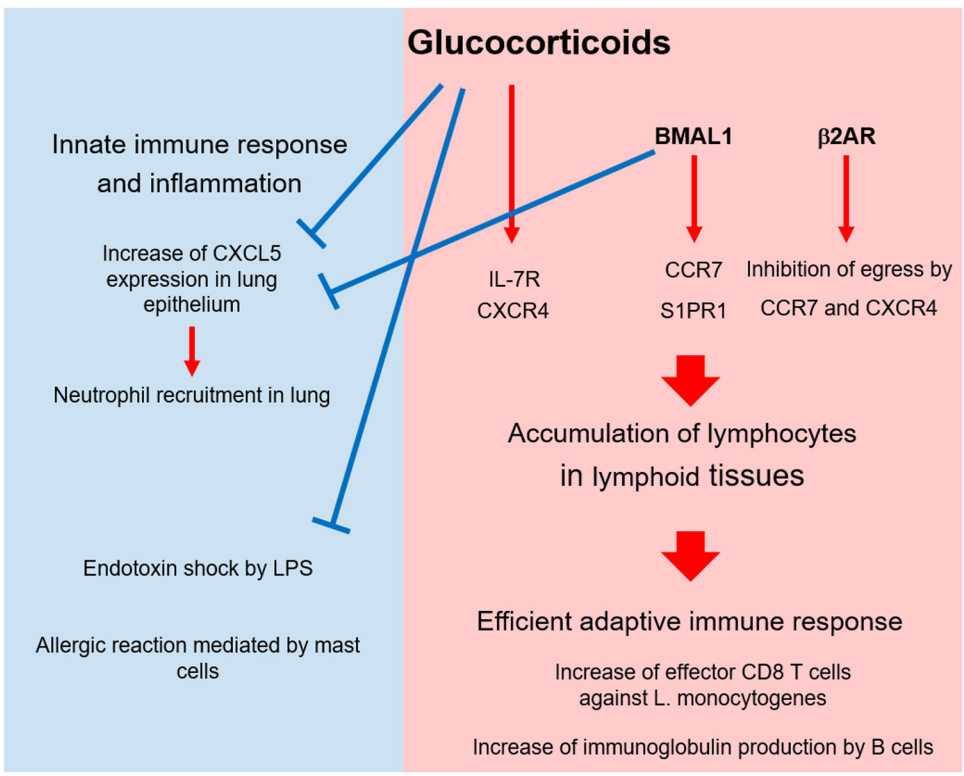

FIGURE 2 | Circadian control of immunity by glucocorticoids and other factors. At the active phase, glucocorticoids (GCs), and BMAL1 suppress CXCL5 expression in lung epithelium and suppress neutrophil recruitment. In addition, GCs alleviate the endotoxin shock stimulated by LPS and the allergic response mediated by mast cells in skin and lung. As immune-enhancing effects, GCs, BMAL1, and β2 adrenergic receptor (β2AR) trigger T cell migration to and retention in lymphoid tissues by enhancing the expression and function of chemokine receptors at the active phase. This accumulation augments T cell-mediated immunity such as anti-bacterial response, B cell activation, and autoimmunity.

contribute to some extent to the T cell response rhythm, but T cell-intrinsic rhythms are the main mechanism involved. Taken together, the circadian rhythm of T cell reactivity seems to show different time courses depending on situations. Thus, more studies will be needed to clarify the mechanisms of the circadian rhythm of immunity.

CONCLUSION

Generally, GCs suppress inflammation and trigger the descending phase of the circadian oscillation of innate immune responses. By contrast, GCs drive the circadian change of T cell distribution and response by inducing the expression of cytokine and chemokine receptors (**Figure 2**). Because other circadian factors such as clock genes and adrenergic signals promote the circadian cycles of an immune response, GCs might cooperate with them to enhance the immune response at the active phase. In addition, because GCs have the potential to enhance permissive effects, they might play a major role in controlling the circadian rhythm of immunity. Because infection risk increases during the active phase, it is reasonable that GCs and other factors induce T cells to home to lymphoid tissues and prepare for an immediate response against infection. Thus, when the circadian rhythm of GCs is disrupted, immune function might be impaired. Conversely, because GCs have the potential to enhance Th2 cell-mediated responses and allergies, their abnormal production might cause excessive Th2 response and allergy. In addition, GCs might affect the diurnal rhythm of asthma symptoms via mast cells and Th2 cells. Future work should focus on revealing the mechanism through which the irregular production of GCs causes immune dysfunction and inflammation such as allergy and autoimmunity.

AUTHOR CONTRIBUTIONS

AS wrote the first draft of the manuscript. KI modified, revised, and approved the submitted version. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by JSPS KAKENHI Grant Numbers 16K15288 and 20K21525 (KI), and 18K15184 and 20K16280 (AS). KI was supported by a grant from the Naito Foundation and Uehara Memorial Foundation. AS was supported by the Shimizu Foundation for Immunology and Neuroscience grant for 2016, grant from the Takeda Science Foundation, and Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical care for 2018. It was also supported by the Joint Usage Research Center Program of the Institute for Frontier Life and Medical Sciences, Kyoto University.

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