OXFORD JLB JOURNAL OF LEUKOCYTE BIOLOGY

Journal of Leukocyte Biology, 2023, **113**, 555–566

https://doi.org/10.1093/jleuko/qiad038
Advance access publication 31 March 2023
Review Article

The circadian neutrophil, inside-out

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Abstract

The circadian clock has sway on a myriad of physiological targets, among which the immune and inflammatory systems are particularly prominent. In this review, we discuss how neutrophils, the wildcard of the immune system, are regulated by circadian oscillations. We describe cell-intrinsic and extrinsic diurnal mechanisms governing the general physiology and function of these cells, from purely immune to homeostatic. Repurposing the concepts discovered in other cell types, we then speculate on various uncharted avenues of neutrophil–circadian relationships, such as topology, metabolism, and the regulation of tissue clocks, with the hope of identifying exciting new avenues of work in the context of circadian immunity.

Keywords: myeloid, neutrophils, granulocytes, circadian, cell trafficking, immune response

1 Introduction

1.1 Circadian oscillations

The Earth provides a hospitable home for millions of species. The rotation around its axis and the sun generates a mixed landscape of ecosystems and rhythms. Attuned to Earth's periodicities, species evolved oscillations relevant to their specific demands. Notably, circadian rhythms, the adaptations to daily changes in sunlight, temperature, or nutrient availability,[1] prevail in nearly all organisms and control a wide range of vital functions. In zones that lack diurnal light/dark oscillations, such as the arctics, organisms lack classical circadian rhythmicity, highlighting the importance of environmental cues for the evolution and maintenance of intrinsic circadian oscillations.[2]

The emergence of circadian rhythmicity coincides with the evolution of photosynthesis in cyanobacteria.[3,4] In these photosynthetic prokaryotes, diurnal oscillations primarily serve to achieve temporal gating of metabolic activity during the diurnal cycle. In higher eukaryotes, circadian timekeeping governs a wide range of processes and features mechanistic complexity.[5] The mammalian clock machinery is governed by a transcriptional/posttranslational feedback loop that involves 2 prominent transcription factors, Clock and Bmal1,[6] which form a heterodimer and prompt the expression of a diverse repertoire of genes (Fig. 1a). The binding of Clock/Bmal1 complex to enhancer boxes, induces the transcription of repressor proteins period (Per) and cryptochrome (Cry). The emergence of these repressors depletes the supply of Clock/Bmal1 heterodimers and, eventually, the pool of repressor proteins, thereby resetting the circadian clock.[5] Certain families of nuclear receptors—namely, ROR and REV-ERB—are involved in fine-tuning the clock activity by positive or negative regulation of Bmal1 activity, respectively.[7]

The maestro of the mammalian circadian clock is the suprachiasmatic nucleus (SCN) region of the hypothalamus, also referred to as the central clock.[8] Instructed by the extrinsic photic cues, the SCN synchronizes the peripheral clocks that are present in parenchymal and circulating cells. The internal clock of the SCN is autonomous and perseveres even in isolation,[9,10] while the oscillations in peripheral tissues rapidly wear off in the absence of SCN input, as shown by SCN injury studies.[9,11] Nevertheless, the peripheral clocks also exhibit autonomy and align with cell- or tissue-specific functionality.[12,13] These peripheral clocks can be additionally synchronized by light-independent periodic cues, such as feeding/fasting cycles[14] or immune cell infiltration.[15] Despite the inherent autonomy of circadian rhythmicity, periodic light/dark exposure is a fundamental entrainment factor that maintains organismal health. Indeed, circadian irregularity in night-shift workers, social jet-laggers, and even visually impaired individuals substantially affects susceptibility to metabolic diseases, malignancies, and infections.[16] The impact of the circadian clock on organismal well-being is to a certain extent indirect, but the components of the core clock also directly regulate immunity, as we will discuss in the following sections.

1.2 The circadian immune system

A plethora of immune-related circuits display circadian rhythmicity.[17] For instance, the number of circulating immune cells in mice and humans oscillates during the 24-h cycle, and most subsets peak at the rest phase of the respective species (i.e. day for mice, night for humans). Earlier studies demonstrated that the magnitude of the immune response, which influences the susceptibility of individuals to infection, is dictated by the time of pathogen exposure. Mice challenged with either lipopolysaccharide or gram-positive bacteria at night exhibit higher mortality,[18–20] a phenomenon that was later attributed to the oscillations of serum cytokine levels, which strongly promote an inflammatory response.[21,22]

Although critical, the example of serum cytokine fluctuations is a minute example of immunological processes governed by

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Received: February 6, 2023. **Revised:** March 14, 2023. **Accepted:** March 16, 2023. **Corrected and Typeset:** April 17, 2023

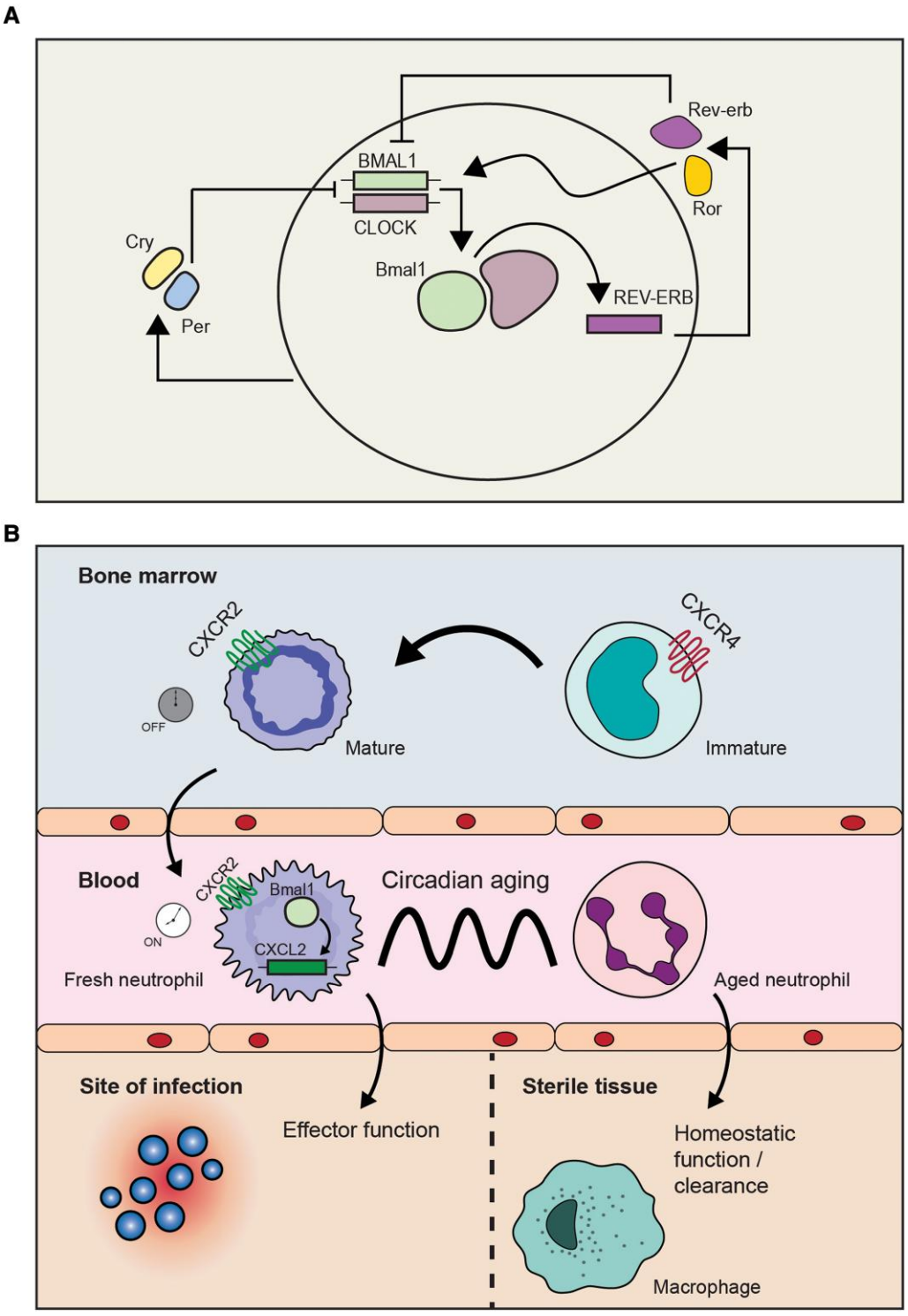

Fig. 1. The core clock and neutrophil aging. (a) The core circadian clock in mammals relies on strict transcriptional/translational regulation. The heterodimer of Bmal1 and Clock, 2 activator transcription factors, induces the expression of many clock-controlled genes, which are involved in regulating key intracellular processes. In doing so, the BMAL1-CLOCK heterodimer also promotes transcription of its own repressors—namely, Period and Cry—as well as the nuclear proteins ROR and REV-ERB, which are collectively involved in resetting and fine-tuning the circadian clock. (b) Neutrophil release from the bone marrow is regulated by antagonistic chemokine signaling through the receptors CXCR4 and CXCR2. During maturation, neutrophils gradually upregulate CXCR2 on the cell surface and become more receptive to its ligands CXCL1 and CXCL2, which promote entry to circulation. Neutrophils recently egressed from the bone marrow, termed "fresh" here, efficiently migrate to inflamed or infected tissues. At steady state, CXCR2 signals drive the circadian aging of circulating neutrophils through cell-intrinsic oscillations in Cxcl2 gene expression, governed by BMAL1. Aged neutrophils subsequently move from circulation into healthy, sterile tissues for elimination or to execute homeostatic functions.

the circadian clock and core clock components, which include cell differentiation, recruitment, clearance, metabolism, and cell-to-cell communication. Numerous studies in mice point to Bmal1 as a prominent regulator of immunity, for instance, through controlling chemokine availability and cell-intrinsic and extrinsic migratory behaviors. The entry and exit of dendritic cells

and lymphocytes into the lymph node rely on Bmal1 oscillations in both immune cells and the partner stromal and vascular cells. Many molecules involved in immune cell recruitment to the lymph nodes, such as CCR7, CCL21, Lyve-1, S1PR1, and integrins, exhibit circadian rhythmicity, which in turn leads to oscillations in the associated adaptive immune response.[23,24] In various other tissues and contexts, Bmal1 intrinsically[25] or extrinsically[26] controls myeloid cell recruitment through chemokine–chemokine receptor interactions.

Recently, REV-ERBa, a member of the REV-ERB nuclear receptor family, has emerged as another clock component critical for the regulation of both innate and adaptive immunity. A number of studies have demonstrated that REV-ERBa restricts inflammasome activity and other inflammatory pathways in the context of infection,[27] endotoxin challenge,[7] neuroinflammation,[28] and colitis.[29] The culminating evidence is that REV-ERBa is responsible for inhibiting NLRP3 as well as NF-κB signaling, both of which are strong inflammatory intermediates.[28,29] Within the adaptive immune cell compartment, REV-ERBa and the ROR family of nuclear receptors are involved in T helper cell differentiation.[30–32] Seemingly potent anti-inflammatory effects of REV-ERBa led to a series of studies exploring the therapeutic potential of REV-ERBa agonists,[33–37] turning the spotlight on the circadian clock as a therapeutic target.

1.3 The circadian life of neutrophils

Neutrophils are short-lived myeloid cells with critical roles for host protection in all vertebrates.[38] Their short life span, combined with high abundance in the circulation, creates a massive production demand, which is primarily met by the bone marrow.[39] Each day, the bone marrow produces billions of neutrophils that are subsequently released into the bloodstream and cleared off in various tissues.[40–42] Using fate reporter mouse models (using an inducible Ly6G^{CreERT2} driver), we estimated the half-life of neutrophils in circulation to be around 10 h, within a similar range as seen in the spleen, lung, and liver, but shorter than in others such as the skin and bone marrow.[42] Pathologic environments influence the life span of mature neutrophils, at least in part by promoting survival or proliferation, as seen in cancer,[43,44] *Staphylococcus aureus* skin infections,[45] and hepatic injury.[46] In humans, the life span of neutrophils in the circulation is still debated, with a broad range of values reported. Based on deuterium labeling in vivo, more recent studies proposed a life span of circulating neutrophils of 5.4 d,[47] which was later recalculated to be 38 h.[48] The large discrepancy is likely the result of a different choice of experimental and mathematical methodology. The seemingly short and rhythmic lifetime predisposes neutrophils to circadian regulation, which likely evolved to fine-tune this periodic production-elimination pipeline, as well as diverse cellular functions.

Neutrophils mature in the bone marrow and enter the circulation as postmitotic cells, a process guided in mice by the activity of 2 antagonistic receptors, CXCR2 and CXCR4[49] (Fig. 1b). Signaling through CXCR4, via its ligand CXCL12, favors the retention of neutrophils in the bone marrow, while the ligands for CXCR2—namely, CXCL1 and CXCL2—stimulate neutrophil egress.[49] Release of neutrophils into the bloodstream is subject to circadian gatekeeping, through rhythmic production of CXCL12 by bone marrow stromal cells.[50,51] The circadian drop in the CXCL12 levels, combined with an increase in CXCR2 expression on mature neutrophils, shifts the balance between the opposing chemokine signals toward cell egress.[49] Consistently, CXCR4-deficient neutrophils exhibit reduced bone marrow retention and loss of rhythm in circulation in mice.[52] Neutrophil–vasculature interactions in the circulation along with subsequent trafficking into tissues are governed by the core clock and the sympathetic nervous system.[53]

Mouse studies have provided valuable insights into the specifics of the circadian timekeeping of neutrophils. The active release of neutrophils from the bone marrow occurs between zeitgeber times (ZT) 17 and ZT5, a period when circulating neutrophils are predominantly "fresh" (a term that refers to their recent immigration into blood).[52,54] Over time, circulating neutrophils in mice experience phenotypical and functional changes indicative of cellular aging (Fig. 1b). These "aged" neutrophils are distinguished from their "fresh" counterparts on the basis of low CD62L and high CXCR4 expression. A similar pattern in cell surface expression diurnal dynamics of these receptors is also observed in human-derived neutrophils.[52] Indeed, nighttime-sampled neutrophils (8 PM–8 AM) expressed higher levels of CD62L and lower CXCR4 compared with daytime cells.[52] We emphasize, however, that expression of these markers is not sufficient to categorize "aging" as this can only be evaluated in the context of circadian dynamics.

The circadian patterns of neutrophils rely on external and internal cues.[55] The internal clock is particular in that it can be considered more a "timer" than a bona fide clock. Indeed, all the circadian transformations observed in circulating neutrophils in mice occur in a matter of hours before the cell is eliminated,[52] and therefore individual neutrophils only see 1 diurnal cycle rather than oscillate over several cycles as other cells do. Another distinctive aspect of the circadian neutrophil is that circadian aging only appears to start once the cells move into the circulation, suggesting that the internal clock of neutrophils is kept off while neutrophils are in the bone marrow, in consistence with the high levels of CXCL12 and CXCR4 signaling (which blocks neutrophil aging) in this organ.[52] Finally, only a few hours after mobilization, aged neutrophils infiltrate tissues, where they can be reprogrammed to acquire nonimmune functions.[42] An interesting question moving forward will be to address whether the neutrophil clock stops "ticking" or neutrophils simply shift to a different noncircadian program once in tissues.

In mice, CD62L^{lo} CXCR4^{hi} aged neutrophils peak in blood around ZT5 and subsequently traffic into naive tissues for clearance.[40] Analysis of human-derived blood samples demonstrated that the number of circulating aged-like neutrophils follows similar diurnal cycles, with only a slight shift of the peak value to the early afternoon (4 PM).[52] This rhythmic nature of neutrophil entry in various organs begs the question of whether these cells are involved in the regulation of tissue-specific peripheral clocks.[40] In multiple tissues, rhythmic neutrophil seeding has been shown to influence certain tissue-specific programs.[15,40] Neutrophils entering the intestine, for instance, are cleared in this tissue by macrophages, which in turn modulate cytokine production and, indirectly, hematopoiesis in the bone marrow.[40] In the lungs, neutrophils have been shown to control approximately a quarter of the circadian gene expression, which was significantly disrupted upon systemic neutrophil depletion. A closer look into the circadian programs in the lungs affected by neutrophils revealed processes involved in cancer metastasis, which was confirmed using the B16F1 metastasis model.[40] Similarly, neutrophil infiltration into the liver was linked to an increase in gene expression of *Bmal1* and *Clock*, as well as hepatocyte metabolic activity.[15] Thus, circadian regulation of neutrophils extends beyond mere inflammatory processes and may imprint diurnal dynamics in the physiology of the tissues that they infiltrate, but the full extent of this temporal control remains to be investigated.

2 Circadian properties of neutrophils

2.1 Molecular regulation

The generation of transgenic animals with selective Bmal1 deletion in neutrophils ($Arntl^{DN}$) revealed various physiological programs governed by the cell-intrinsic neutrophil clock.[40,52] For example, diurnal oscillations in the neutrophil transcriptome are susceptible to Bmal1 deletion[52,56] and govern a wide range of immune-related transcriptional programs, such as cytokine signaling, toll-like receptor signaling, and cell migration. Notably, this cell-intrinsic aging program is controlled by CXCR2 signaling through BMAL1-dependent autocrine CXCL2 production. Gene expression of Cxcl2 is significantly downregulated in $Arntl^{DN}$ mice, and both these mice and mice that lack the receptor for the chemokine ($CXCR2^{DN}$) lose the circadian pattern of neutrophil aging. In contrast, CXCR4-deficient neutrophils exhibit constitutive aging, a phenotype consistent with the opposing effects of this receptor on CXCR2 signaling. Importantly, the circadian-gated transcriptional regulation of Cxcl2 facilitates neutrophil clearance from the circulation into tissues, where these cells can serve as a new set of eager troops against imminent pathogen exposure, and indeed excessive neutrophil aging in $CXCR4^{DN}$ mice correlates with resistance to fungal infection.[52]

While BMAL1 and CXCL2 evidently regulate circadian oscillation of gene expression in neutrophils, these cells rarely resort to de novo protein synthesis, which is cost-ineffective considering their short life span. Instead, neutrophils possess an extensive arsenal of proteins stored in their granules, readily mobilized upon pathogen encounter.[57] Thus, it is not surprising that cell-intrinsic circadian oscillations in neutrophils extend to the regulation of the proteome. Indeed, nighttime mouse neutrophils, which have been just released from the bone marrow, are enriched in proteins associated with granules, neutrophil extracellular trap (NET) formation, and cell migration.[56] In contrast, daytime neutrophils, which have been circulating for hours and are thus considered aged, lose their granular cargo along the way.[56] For instance, the decline of granule proteins in the neutrophil proteome correlates with the elevation of elastase activity in plasma, corroborating the steady-state discharge of granules into circulation.[56] The loss of granule content, similar to the transcriptomic changes described previously, is under the control of the Bmal1-driven internal clock, and in vivo and ex vivo analyses showed that "fresh" or night-like neutrophils produce more NETs as opposed to "aged" or day-like cells.[56] Interestingly, similar observations were made in human neutrophils; granule density and granule protein content follow diurnal cycles, reaching a degranulation maximum during the afternoon.[56] Consistently, fresh-like neutrophils in the early morning exhibit higher NET-forming capacities compared with aged-like cells in the afternoon.[56] This fascinating concept of circadian functional oscillations underlies a protective mechanism that we speculate serves to curb neutrophil-mediated tissue and vascular damage. More specifically, neutrophils that enter tissues during the daytime in mice possess less granule content and are therefore less toxic for the tissues, a notion that is in line with the increased severity of acute lung injury at night. Further studies are needed to explore the extent of circadian regulation imposed on the neutrophil proteome. Since neutrophils rely massively on their protein-based armament for the execution of both canonical (i.e. inflammatory) and noncanonical functions, the oscillations in the proteome are likely to have far greater implications in organismal physiology that are yet unclear.

Other granulocytes such as eosinophils, basophils, or mast cells could potentially employ similar disarming mechanisms. However, the evolutionary selection pressure on neutrophil disarming has been conceivably higher given their high abundance in the circulation. Since both basophils and mast cells are mostly tissue resident, degranulation in circulation is not likely to serve as a viable disarming mechanism for these cells. Nevertheless, they could still employ other ways of eliminating their potentially harmful cargo, which is often associated with pathology in type 2 immune diseases.[58]

2.2 Cellular topology and migration

Not only is the molecular profile of mouse neutrophils subjected to aging, but the topology of the cell and internal organelles also undergoes circadian alterations, starting with the actin cytoskeleton and the global shape of the plasma membrane.[52] In general, the intracellular properties of immune cells have been a neglected area of research, and we provide here an overview of how circadian patterns impinge on the cellular structure and function of neutrophils.

During circadian aging, circulating neutrophils lose their microvilli concomitantly with the delocalization of the actin filaments from the cortex to the inner cytosolic part of the cell.[52] This topological transformation of the plasma membrane constrains the neutrophil to adapt their mode of extravasation. The high density of microvilli at the surface of fresh neutrophils enables a classic mode of migration, comprising the 4 main steps of adhesion, rolling, arrest, and diapedesis (Fig. 2b). Because microvilli are fundamental to present ligands for endothelial selectins away from the cell surface and onto the vascular wall,[59] the collapse of these structures renders aged neutrophils less sticky and able to roll under the shear forces present in the bloodstream and instead leads to sudden arrest upon contact with the endothelium and immediately initiate their transmigration to the perivascular tissue[52] (Fig. 2b). This switch in the preferential mechanism of neutrophils to cross the endothelium barrier is partly explained by the fact that the loss of microvilli changes the repertoire and topological distribution of adhesion molecules at the cell surface. Aged neutrophils exhibit a higher density of integrins such as LFA-1, Mac-1, PECAM-1, CD44, VLA-4, and ICAM-1, in comparison to fresh neutrophils[60] (Fig. 2a). Moreover, the b2 integrin Mac-1 expressed by aged neutrophils exhibits higher binding affinity to ICAM-1 relative to the younger cells, suggesting that in addition to being upregulated during circadian aging, Mac-1 undergoes conformational changes that accentuate its binding affinity to the endothelium barrier.[60] Interestingly, adhesion molecules at the surface of endothelial cells have been shown to oscillate in a circadian fashion,[61] with a higher expression of VCAM-1 and ICAM-1 during the dark phase. Overall, neutrophils and endothelial cells both undergo circadian alterations that culminate in enhanced infiltration of naive tissues by aged neutrophils, a property that may be fundamental in the organismal distribution and physiological function of these cells.

NETosis, the active release of chromatin and associated proteins, enables neutrophils to entrap microorganisms and to prevent their dissemination. This process relies in part on the dynamics of the actin cytoskeleton.[62] An early step of NETosis is the translocation of different proteins into the nucleus; for instance, elastase promotes chromatin decondensation and equips the DNA with proteases to kill the targeted pathogen. Translocation of elastase from the cytosol to the nucleus requires intact dynamics of the actin microfilaments network.[62] Studies that compared the NET-forming capacities of fresh and aged neutrophils in mice revealed that both types of neutrophils are

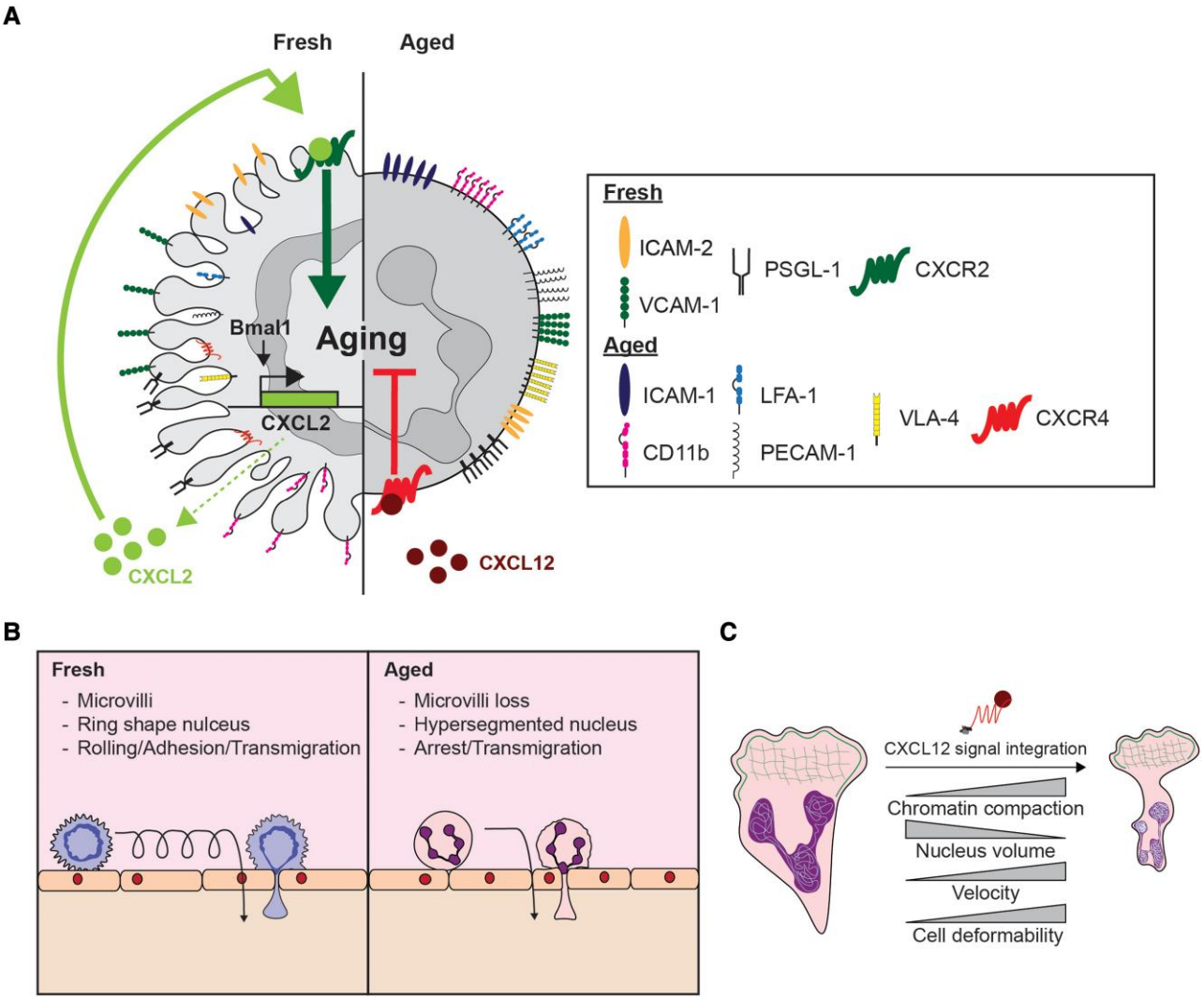

Fig. 2. Cellular topology and neutrophils migration. (a) Once mobilized into blood, fresh neutrophils undergo BMAL1-dependent aging. Nuclear accumulation of BMAL1 promotes the expression of Cxcl2, which in an autocrine manner drives neutrophil aging. At the morphological level, aging promotes microvilli collapse, which leads to the redistribution of integrins and other adhesion receptors. Circadian aging of the cell is complemented by increased CXCR4 expression on the cell surface. (b) The morphological modifications of the plasma membrane as well as the redistribution of adhesion molecules drive a shift in the mode of extravasation. High microvilli density in fresh neutrophils makes them sticky to the endothelial wall and favors rolling on selectins. In contrast, aged neutrophils rely more on integrins now exposed at the cell surface to promote sudden arrest and diapedesis. (c) During circadian aging, neutrophils may undergo topological changes but also the nuclear envelope. In aged neutrophils, the nucleus is multilobulated, thereby facilitating locomotion through small pores in 3D environments where mechanical constraints are high, such as in tissues. Once embedded within tissues, aged neutrophils might migrate toward $CXCL12^{+}$ niches to be reprogrammed. CXCL12 signaling additionally provokes changes in nuclear topology; the chromatin undergoes epigenetic modification that drives nuclear condensation, reduced nuclear volume, and increased cell deformability.

similarly prone to undergo NETosis but exhibit distinct responsive capacities according to the location and/or stimulus. For instance, aged neutrophils exhibit higher NET formation in the context of tumors[63] or sickle cell disease,[64] whereas fresh neutrophils are more efficient at releasing their chromatin in the context of ischemia-reperfusion or acute lung injury.[56] Thus, the debate is still open on how aging influences NET formation and therefore how alterations in cortical actin polymerization affect the global NET-forming capacities of neutrophils.

The actin cytoskeleton also influences the morphology of the nucleus by interacting with the linker of nucleoskeleton and cytoskeleton (LINC) complex.[65] The LINC is made of nesprin and SUN protein families that traverse the nuclear envelope. Although poor in LINC molecules, the neutrophil nucleus is subjected to mechanical constraints by the actin microfilaments.[66] In cells expressing high levels of lamin A/C, such as dendritic cells, nucleation of the actin cytoskeleton around the nucleus is required for cellular migration through small pores.[67] The actin network exerts pressure forces on the nucleus that disrupt the lamin A/C mesh and reduce nuclear rigidity. However, the ARP2/3 complex, which mediates nucleation of actin filaments around the nucleus, is dispensable for HL-60–derived human neutrophil migration through small constrictions.[67] Altogether, those data suggest that alterations in perinuclear actin fibers are unlikely to facilitate the extravasation of aged neutrophils into tissues. However, this concept is challenged by findings linking the actin–nuclear envelope interactions via myosin-1f (Myo1f) with neutrophil transmigration through small pores.[68] Likewise, Myo1f has been associated with the navigation of neutrophils within 3-dimensional (3D) microenvironments, where the mechanical constraints are

high. In both contexts, the actin–Myo1f and nuclear envelope complex helps to reduce the nuclear volume and increase its deformability. Thus, perinuclear accumulation of actin microfilaments may prepare aged neutrophils to migrate in 3D environments. In conclusion, the actin cytoskeleton is important for the locomotion of neutrophils despite their low lamin content and may therefore modulate the ability of neutrophils to differentially migrate in 3D microenvironments at different times of day.

2.3 Locomotion in 3D environments

Fresh or aged neutrophils, although exhibiting drastic differences in surface topology, are both capable of transmigrating into tissues and migrating in 3D microenvironments. However, they follow 2 distinct paradigms of extravasation and recruitment: fresh neutrophils are more prone to be recruited into inflamed tissues, whereas aged neutrophils are more efficient at infiltrating naive tissues.[52] This implies that both types of neutrophils are most likely to navigate in different environments, following a distinct gradient of chemokines and moving toward different structures within a tissue. Hence, they may be equipped accordingly. For instance, in the healthy lungs of mice, aged neutrophils accumulate in the periphery of large blood vessels, following a gradient of CXCL12.[40,42] In contrast, in injured skin, recruited neutrophils move toward areas of inflammation and must cross regions of disrupted extracellular matrix. Thus, the different migratory properties of fresh and aged neutrophils might enable partition, in time and space, of cells with different properties: one devoted to responding to perturbations in tissues and another one devoted to supporting organ homeostasis, thereby avoiding the potential perils of inflammatory fresh neutrophils in healthy tissues.[52,56]

This model, while speculative, aligns with studies conducted in mice and shows that the nature of chemokine gradients followed by neutrophils and their mode of locomotion relies on topological adaptations.[69,70] For instance, in the injured skin, neutrophil migration toward the wounded site results from a 2-stage process that relies on the dynamics of actin microfilaments.[69] In a first "search" phase, neutrophils sense their microenvironment to capture chemotactic signals. This necessitates the polarization of the actin cytoskeleton at the front edge of the cell that concentrates chemokine receptors at this front edge. In the second "run," phase neutrophils abruptly stop their random movement, reorientate their leading edge, and speed up toward the source of chemokines. This latter stage is dependent on the actin flow dynamics from the front to the back edge and the actin–myosin contractile structures accumulating at the back of the neutrophil.[69] Thus, during wound-oriented migration, neutrophils (and possibly other leukocytes) initially sense the gradient of chemokines and then build a high-pace conveyor belt to rapidly reach the target site. Efficient actin nucleation increases the area of exploration.[69] Thus, equipped with a dense cortical network of actin microfilament, fresh neutrophils appear to be more adapted to sense and quickly respond to inflammatory chemotaxis cues than their aged counterpart.

Upon homeostasis, extravasating neutrophils experience and respond to a gradient of CXCL12 molecules via the G-protein coupled receptors CXCR4 and atypical chemokine receptor 3. Subsequently, a signal is sent to the cell through the protein kinase A to promote the dimethylation of the H4K20 histone site, thereby accentuating the chromatin compaction and protecting the chromatin from migration-induced breaks. This change in chromatin topology leads to a nuclear volume reduction and potentiates neutrophil motility in confined spaces[70] (Fig. 2c).

In conclusion, the nature of the chemokine gradient regulates the type of topological transformations required for a successful migration and might explain the morphological differences adopted by neutrophils at difference phases of the diurnal cycle.

2.4 The circadian nucleus

The nuclear topology of neutrophils undergoes circadian modifications in mice[54] and humans[71] by transforming from a ring shape to a hypersegmented profile. While the molecular basis of this circadian alteration is not understood, several studies addressing the molecular structure of the murine and human nuclear envelope may provide some insights. The multilobular shape of the nucleus is progressively acquired during granulopoiesis (i.e. the maturational process from a common granulocyte–monocyte progenitor into a mature neutrophil). This distinct morphology is caused by the lamin B receptor (LBR) and the lamin composition of the nuclear skeleton, which is rich in lamin B2 and low in lamin A/C.[66,72–74] During neutrophil maturation, LBR and lamin B2 expression follows opposite dynamics in comparison with lamins A/C and B1, in that they are progressively upregulated. LBR expression is under the regulation of CEBP/e,[75,76] a transcription factor known to be essential for neutrophil development,[77] and accordingly, patients with the Pelger-Huët anomaly (PHA) who carry mutations in the gene encoding LBR feature an abnormal hypolobulated nuclear in neutrophils.[78] Interestingly, LBR affects the nuclear topology and chromatin organization of human neutrophils in a genotype-dependent manner, as homozygous carriers exhibit a lobeless ovoid nucleus, and suggesting that LBR plays a dose-dependent role on the degree of segmentation. Thus, while the molecular basis of the circadian alterations in nuclear morphology are not understood, they are likely to be influenced by changes in the nuclear envelope proteins and to affect fundamental aspects of neutrophil biology.

The modification of the nuclear shape during neutrophil aging likely affects fundamental aspects of the cell biology. Indeed, the nuclear morphology of neutrophils has been observed to influence different canonical tasks, including chemotaxis,[70,79] as well as the chromatin organization,[80] further influencing gene expression.[81] But how might changes in the nucleus affect the capacity of aged neutrophils to reach tissular niches?

Progressing in the tissue parenchyma requires morphological adaptability and, notably, a nucleus that can deform and bear mechanical stress without breaking.[82] Neutrophils are naturally equipped with a nucleus of lower stiffness in comparison with other leukocytes due to the aforementioned composition of their nucleoskeleton largely devoid of lamin A/C.[66] However, low lamin expression makes the cell prone to nuclear envelope rupture in scenarios of high tension, as seen in laminopathies.[83,84] Physical stress on the nucleus may be particularly high during migration in confined environments, and hence it is likely that to circumvent this biological hurdle, neutrophils feature high heterochromatin at the periphery of the nucleoplasm.[85] Similarly, it is tempting to speculate that increasing the tortuosity of the nuclear envelope, as seen in aged neutrophils, could reduce the volume occupied by the nucleus and therefore lower the exposure of the nucleus to the mechanical stress imposed by confined spaces, thereby protecting the nuclear envelope and chromatin integrity. Elegantly illustrating this model, studies have shown that in response to chemokine gradients, neutrophils adapt their chromatin topology to reduce the nuclear volume and increase its deformability.[70] While these analyses did not address the circadian alterations in nuclear topology, it is intriguing that

neutrophils upregulate CXCR4 during aging[54] and migrate toward CXCL12-enriched areas in the lung.[42] Another potential value of a multilobular nucleus is hinted from recent findings demonstrating that the nucleus guides 3D migration of cells through paths of least resistance.[86] Although this study was conducted on "mononuclear" cells, it is possible that each segment of the neutrophil nucleus acts independently to cover more possible trajectories. In this light, it would be very informative to compare the migration of neutrophils from PHA patients-derived vs healthy patients in an artificial 3D environment that mimics the mechanical constraints of tissues. Overall, we propose that the circadian transformation of nucleus topology renders aged neutrophils more capable of migrating in 3D environments, consistent with their preferred location into naive tissues.

Contrasting with migration, the influence of nuclear topology on other effector functions of neutrophils, including release of reactive oxygen species, cytokines, or NETs, is less evident.[66] Several studies have addressed this question by analyzing the functional capacities of PHA-derived neutrophils or LBR-deficient mouse neutrophils. Interestingly, although essential for nuclear morphology, LBR is not required for efficient killing of *S. aureus* in mice.[75] Likewise, neutrophils from patients with PHA do not present defects in phagocytosis or chemotaxis in comparison with those from healthy individuals.[87] Nevertheless, LBR protein expression and nuclear topology can influence effector functions depending on the species. Indeed, in an ex vivo model of mouse neutrophil differentiation, full deficiency in LBR protein synthesis compromises chemotaxis and oxidative burst but not phagocytosis.[79] In conclusion, circadian alterations in nuclear morphology are likely to influence fundamental aspects of immune and non-immune roles of neutrophils, particularly in tissues or at sites of inflammation or infection.

3 Unconventional features of the circadian neutrophil

It is unclear whether the set of circadian transformations that neutrophils are subjected to includes modification of the chromatin architecture. However, the recent advances on nuclear biology discussed above make it conceivable that chromatin topology is affected by the nuclear deformation associated with circadian aging or during tissue extravasation and migration. Similarly, the fact that neutrophils upregulate the cell surface expression of CXCR4 during circadian aging,[54] that the CXCL12/CXCR4 signaling axis promotes the homing of neutrophils into their tissue niche,[42] and that CXCL12 promotes chromatin remodeling in neutrophils[70] suggest that neutrophils in tissues undergo nuclear deformation and in epigenetic landscape remodeling as they follow CXCL12 gradients. Last, although largely transcriptionally inactive at this stage, aged neutrophils have been shown to engage in new transcriptional programs when embedded within tissues, a finding also suggestive of chromatin remodeling.[42] Despite their poor transcriptional rates, new technologies[88] should enable dual analysis of chromatin and transcriptome profiles of fresh and aged neutrophils in relevant pathophysiological scenarios. In this line, it will be interesting to evaluate whether the chromatin organization of neutrophils from patients with PHA is altered secondary to the deficiency in LRB protein and whether they are capable of transcriptional reprogramming when embedded in tissue microenvironments.

Neutrophils can eject the nuclear chromatin into the outer environment to trap microorganisms and limit their dissemination. The released chromatin is decorated with granule-derived proteins, including elastase, cathepsin G, proteinase-3, and myeloperoxidase, all of which are devoted to killing the entrapped microorganisms.[89,90] Depending on the stimulus initiating the release of NETs, the location, and the inflammatory cost of such event, different regulatory mechanisms for NET formation can be distinguished.[91] The timing and type of NET formation are associated with circadian oscillations as indeed circadian aging compromises the capacity of neutrophils to release chromatin, as shown in vitro upon PMA activation and in vivo in a model of ischemia-reperfusion.[56] This reduction in NET-forming capacities was explained by the gradual loss of granules and their content,[56] which are needed for chromatin decondensation and nuclear disintegration.[92] Conversely, in the context of sickle cell disease[64] or in cancer,[63] aged-like neutrophils are predisposed to undergo NETosis and thereby promote vaso-occlusive events or tumor cell metastasis, respectively. The reasons underpinning this dichotomy in NETs release capacities remain to be elucidated, but it is possible that differences in defining neutrophil aging, in location (i.e. tissue,[56] circulation,[64] or microcirculation[63]), or inducting stimulus (i.e. hypoxia[56] vs erythrocyte homeostasis[64] or tumor-derived factors[63]) may drive these differences, and it will be important to understand how changes in these factors during the diurnal cycle influence the content and properties of NETs.

Another intriguing aspect of neutrophils that may relate to circadian oscillations is their mitochondrial architecture and metabolism. The mitochondrial architecture oscillates from a tubular to a fragmented morphology by way of fusion and fission, thereby modulating the bioenergetic properties of mitochondria, ATP production, and calcium release, which in turn sustain the nutritional demands of the cells.[93] For instance, when nutrients abound, the mitochondrial network undergoes fragmentation to facilitate mitophagy. Interestingly, the fusion/fission cycle has been found to undergo circadian oscillations[94,95] and to rely on the molecular clock machinery in hepatocytes[96] as well as in cultured skin fibroblasts and brain lysates.[97] In addition to producing energy, mitochondria balance immune functions.[98] Specifically, the NET-forming capacity of neutrophils partly relies on the mitochondrial network and ATP production. Indeed, "fusioned" mitochondria potentiate NETosis.[99] Neutrophil motility and tissue extravasation also depend on the integrity of their mitochondrial pool, as illustrated by the finding that mitofusin 2 (Mfn2)–deficient neutrophils remain trapped inside the vasculature in zebrafish.[100] Likewise, Mfn2-deficient neutrophils cannot extravasate in mouse models of peritonitis.[100] It will be interesting to examine whether mitofusins or other components of the molecular machinery involved in mitochondrial dynamics of neutrophils undergo circadian regulation and the extent to which they regulate their migratory and cytotoxic activity in vivo.

The circadian clock and metabolism of a cell are intimately intertwined and regulate one another.[101] While nutrient intake is known to entrain peripheral clocks,[102,103] the central clock governs catabolic and anabolic reactions that sustain life.[104] Deletion of core clock genes disrupts the balance of glucose, lipid, and amino acid metabolism and impairs the function of tissues with high metabolic activity, such as the liver.[105,106] In myeloid cells, metabolic activity dictates broad functional properties, such as inflammatory activity and cell differentiation.[107,108] Various anchor points between metabolism, circadian clock, and immunity have been studied in macrophages or dendritic cells,[109–112] but whether these mechanisms also apply to neutrophils is not known. For instance, the mechanism of glucose degradation in macrophages influences the differentiation into the M1- or M2-like phenotypes, thereby influencing their

inflammatory or reparative fate, respectively.[113] It has recently been shown that oscillations of antigen processing activity in dendritic cells rely on mitochondrial metabolism, which is regulated by Bmal1 expression.[114] It would be of utmost interest to elucidate the contribution of cell-intrinsic or extrinsic circadian rhythms to neutrophil metabolism and the downstream cellular function.

4 Concluding remarks

Here, we have reviewed and discussed the molecular and topological transformations that circulating neutrophils experience throughout the circadian cycle, a time frame encompassing almost the entirety of a neutrophil's life. We highlight clock-driven adaptations, including the remodeling of transcriptome, proteome, cytoskeleton, and cell nucleus, all of which serve to fine-tune the timing of physiological and immunological programs. We describe the morphological changes in circulating neutrophils and the ensuing functions attributed to fresh and aged cells.[52,70,79]

Despite extensive recent work, the field of circadian neutrophil biology is currently in its infancy, surrounded by more unknowns than knowns, and despite progress, many questions need to be placed in context. For instance, the clock is predominantly studied in circulating neutrophils while its activity within the bone marrow or other tissues is unclear. In line with this, a relevant challenge will be to understand whether reprogramming of the neutrophil clock can occur based on local extrinsic cues (i.e. depending on tissue context). Additionally, we emphasize the importance of defining tissue-specific circadian programs that are driven by the diurnal infiltration of neutrophils, as seen in hepatocyte metabolism.[15] We speculate that the cellular and nuclear topology of neutrophils, both of which are actively restructured to improve motility and function,[52,70] are susceptible to circadian regulation. Live imaging techniques with subcellular resolution, combined with clock-deficient transgenic mouse models,[52] should allow tracking of topological transformations of neutrophils. Delineating topology–clock relationships and other intracellular aspects of the neutrophil will add a precious piece to the neutrophil circadian puzzle.

Finally, because the internal timer separates in space and time (immune "gating") the antimicrobial and proinflammatory functions of neutrophils, and because aged neutrophils can cause severe vascular inflammation, manipulation of this circadian neutrophil timer is of clear clinical relevance. For example, targeting the receptors that regulate this phenomenon, such as CXCR4,[52] may offer therapeutic alternatives for the broad range of diseases where neutrophils are main actors, including infections and cardiovascular diseases. Nevertheless, ideal therapeutic candidates should exclusively target circadian aging of neutrophils and no other physiological processes, which will be a challenging task.

Authorship

S.O., A.O., and A.H. contributed to the writing and editing of this manuscript. S.O. and A.O. designed the figures.

Acknowledgments

We thank all members of our laboratory for discussion and contributions to the concepts included in this revision.

Funding

This study has been possible through grants R01AI165661 from the National Institutes of Health, RTI2018-095497-B-I00 from Ministerio de Ciencia e Innovacion, HR17_00527 from Fundación La Caixa, and FET-OPEN (no. 861878) from the European Commission. A.O is supported by the Swiss National Science Foundation (P500PB-206852). The CNIC is supported by Ministerio de Ciencia e Innovacion and the Pro CNIC Foundation and is a Severo Ochoa Center of Excellence (CEX2020-001041-S).

Conflict of interest statement. A.H. is a paid consultant for Calida Therapeutics. The other authors declare no conflict of interest.

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