Fugue G Minor: Getting the Lymph Node Ensemble Together with Circadian Rhythm

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A fugue is characterized by the systematic repetition of a principal theme in simultaneous melodic lines. In this issue of *Immunity*, Druzd et al. (2017) show that a similar phenomenon occurs in lymph nodes (LNs), in which lymphocyte entry and exit is governed by repetitive circadian rhythms.

Circadian rhythms are oscillatory cycles in biological processes that occur over a 24 hr span (Halberg, 1963). The sleep-wake cycle is one of the most obvious biological phenomena regulated by circadian rhythm and, not surprisingly, the perception of light is one of the factors that synchronizes the master clock via the hypothalamic-pituitary-adrenal axis. At the molecular level, circadian rhythms are governed by the core clock genes, *Bmal1* and *Clock* (Mohawk et al., 2012), which are helix-loop-helix transcription factors that bind to canonical E-Box sequences and promote the expression of clock-controlled genes. Some clock-controlled proteins, such as Per and Cry as well as Rev-erbα and Rev-erbβ, form complexes that negatively regulate the cycle by preventing *Bmal1* and/or *Clock*-dependent transcription, thereby leading to cyclic rounds of expression and repression. Proteins in the clock pathway also influence the expression of non-clock genes, which allows them to regulate a variety of biological responses, including immune responses (Scheiermann et al., 2013). For example, Bmal1 regulates the accumulation of inflammatory monocytes at sites of inflammation by diurnally suppressing the expression of CCL2 and CCL8 chemokines (Nguyen et al., 2013). Similarly, Rev-erbα regulates the diurnal accumulation of T helper 17 (Th17) cells in the intestine by indirectly controlling the expression of Rorγt transcription factor via an intermediary transcription factor, Nfil3 (Yu et al., 2013). In contrast, other studies suggest that despite the cyclic expression of Bmal1 in lymphocytes (Hemmers and Rudensky, 2015), its loss does not dramatically affect T or B cell differentiation, suggesting that lymphocyte-extrinsic circadian rhythms (Gibbs et al., 2014) might be more important in controlling immune responses than lymphocyte-intrinsic circadian rhythms.

In the current manuscript (Druzd et al., 2017), the authors show that the numbers of lymphocytes in the LNs fluctuated throughout the day in a sinusoidal pattern—with cells accumulating to their highest numbers around the start of the active period (dark cycle in mice) and declining to their lowest numbers around the beginning of the resting phase (light cycle in mice) (Figure 1). They also found that this cyclic pattern of cellular accumulation and depletion was maintained when light/dark cycles were temporarily interrupted, but was inverted when mice were continuously housed with a reversed light/dark schedule. Thus, they concluded that lymphocyte entry and/or egress in LNs is controlled by bona fide circadian rhythms.

Naive lymphocytes gain entry to LNs through high-endothelial venules (HEVs) by the sequential engagement of CD62L, CCR7, and LFA-1 on lymphocytes, with their ligands, PNAd, CCL21, and ICAM-1, on HEVs. Thus, the authors reasoned that one or more of these molecules were likely expressed in a circadian fashion. In fact, they found that the expression of both CCR7 and its ligand CCL21 increases from *Zeitgeber* time (ZT)1 until ZT13 (lights on) and then decreases again between ZT13 and ZT1 (lights off) (Figure 1). These times also correspond with times of increased cellularity in the LN and decreased numbers of lymphocytes in the blood, suggesting that cyclic variations in lymphocyte-intrinsic (CCR7) and lymphocyte-extrinsic (CCL21) molecules controlled LN entry.

They next tested whether the egress of lymphocytes from LNs was regulated in a circadian fashion. By cannulating the efferent lymphatics of mesenteric LNs, they found that lymphocyte egress from LNs peaked at ZT9 and was lowest at ZT21—times that are slightly offset from peaks in lymphocyte entry into LNs (Figure 1). Importantly, they also found that the sphingosine-1-phosphate receptor (S1P1R), which controls lymphocyte egress, was expressed on lymphocytes in an oscillatory pattern. Moreover, using mice lacking one allele of the S1P1R or by blocking the activity of the S1P1R using FTY720, they showed that impaired S1P1R function abolished the cyclic pattern of lymphocyte release into the lymph. Thus, they concluded that the cyclic accumulation and depletion of lymphocytes in the LN is controlled at the level of both entry and exit.

To test whether these activities were regulated by a lymphocyte-intrinsic circadian clock, the authors first showed that the expression of the clock genes, *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, and *Nr1d1* were expressed in an oscillatory pattern in lymphocytes (Figure 1). Although the authors only examined gene expression over a 24 hr period, one can infer that the cyclic nature of gene expression repeats every 24 hr as reported in other studies (Haspel et al., 2014). Moreover, they found that lymphocytes lacking Bmal1 failed to accumulate in a cyclic fashion in LNs and that the cyclic variations in the expression of CCR7 and S1P1R on these cells were blunted. In contrast, they found that the levels of S1P in lymph and the expression of enzymes controlling S1P production or degradation did not vary over time.

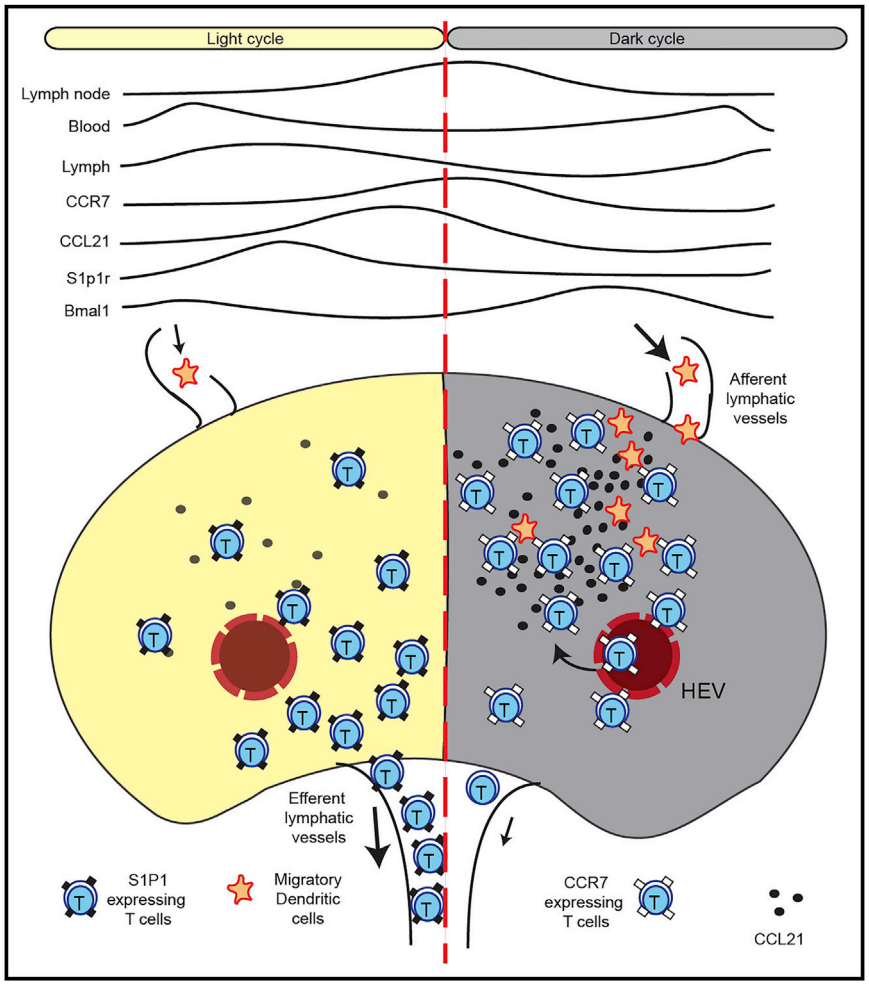

Figure 1. Cell-Intrinsic Circadian Rhythms Control Lymphocyte Recruitment in Lymph Nodes

The figure is divided by time of day into the light cycle (left side) and dark cycle (right side). The cyclic variations in cell number in the LN, blood, and efferent lymph, as well as the cyclic variations in gene expression, are shown schematically throughout the day (top). The effects of these changes on LN cellularity are shown in the schematic below, in which fewer cells enter and more cells leave the LN during the light cycle and more cells enter and fewer cells leave the LN in the dark cycle.

Thus, T cells, B cells and HEVs have cell-intrinsic molecular clocks that orchestrate their coordinated ability to enter and exit LNs.

To test whether the cyclic accumulation of lymphocytes in the LN was biologically important, the authors immunized mice with myelin oligodendroctye glycoprotein (MOG) peptide emulsified with Complete Freund's Adjuvant and pertussis toxin, which normally induces an autoreactive T cell response that leads to spinal cord demyelinization and impaired movement. Strikingly, they found that immunization immediately before the peak of LN cellularity (ZT8) led to a higher clinical score, more severe demyelination, and greater numbers of pathogenic Th17 cells than did immunization just before the nadir of LN cellularity (ZT20). Most importantly, these differences were not observed in mice lacking Bmal1 expression in T cells. Thus, the timing of antigen encounter in relation to the circadian clock has a surprisingly profound effect on both the rate and magnitude of the subsequent adaptive immune response.

LNs recruit rare, antigen-specific lymphocytes from the blood, as well as antigens and antigen-presenting cells from peripheral tissues, and provide specialized domains, such as B cell follicles, T cell zones, and even germinal centers, that support the proliferation of antigen-activated lymphocytes and promote their differentiation into appropriately-activated effector cells. Thus, it makes sense that a mechanism to coordinate the simultaneous recruitment of B and T cells to the LNs at a particular time of day will maximize immune responses at that time. However, the temporal nature of this mechanism also poses some questions. For example, one might assume that antigen emulsified with CFA would be released gradually over a period of days or weeks as observed in other studies (Hailemichael et al., 2013). If so, then one might expect a minor 12 hr delay in the progression of immune responses following vaccination at off-peak times, rather than a dramatic drop in disease severity that persists for weeks. Does this result suggest that antigen is only transported to the LN for only a limited period following immunization and then eliminated before the next arrival of lymphocytes? Alternatively, does the arrival of antigen out of phase with the peak of lymphocyte accumulation affect T cell differentiation in a way that skews them toward a non-pathogenic effector program? At this point, only minimal information is available. Thus, one suspects that these questions will be answered by future studies using experimental antigens and even infectious agents. A more vexing question is why the immune system would evolve a mechanism that leaves the host potentially immunocompromised at particular times of day? It seems unlikely that such a fundamental mechanism would remain if it were detrimental to the host. Thus, the advantages of such as system must outweigh any disadvantages and it remains for us to determine what those advantages might be so that they can be incorporated into vaccine strategies or other types of immune therapy.

The observations of Druzd et al. also have practical implications well beyond our mechanistic understanding of immunity. For example, the NIH is now requiring evidence of reproducibility and rigor in its grant applications and supported publications. Given that these data convincingly demonstrate that encountering antigen at different times of day leads to different outcomes, should we now perform all experimental immunizations and infections at a standard time—let's say 9 AM? Patients are currently seen by physicians throughout the day based on

the limited availability of appointments. Should all vaccinations be administered at 9 AM? Using electronic medical records, hospitals are now beginning to track clinical outcomes of various procedures with time of admittance. However, most studies are interested in how the attentiveness of healthcare workers or shift changes impact clinical outcomes, rather than how the patient's own circadian rhythms might affect a biological response to treatment.

In summary, we now know that cell-intrinsic circadian rhythms alter both the entry and egress of lymphocytes via Bmal1-dependent changes in the expression of homing receptors and ligands on their surface. Despite the relatively modest magnitude of these changes and their short periodicity, they seem to have an outsized effect on subsequent immune responses. Future studies will have to take into account the timing of immunization and infection on the immunological outcome of any particular immune response and determine the mechanisms that temporally control immunity and host defense. Meanwhile, one is left to ponder whether the compositions of Johann Sebastian Bach were influenced by the circadian periodicity of his own immune system.

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Th17 Cells Require You to Chew before You Swallow

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How immunity is regulated at distinct epithelial tissues that vary in microbial occupancy and environmental and tissue specific cues isn't clear. Dutzan et al. (2017) report that mechanical-derived signals, not those from micro-organisms, are key to maintaining interleukin-17-expressing T helper (Th17) cells at the oral epithelia.

Detailed knowledge of $CD4^+$ T helper (Th) cell biology has been gained over the last decades. Initially split in two subsets, Th1 cells and Th2 cells, the discovery of Th17 cells did not contribute just a third effector arm of the $CD4^+$ lineage. Th17 cells are rare in secondary lymphoid organs but enriched at epithelial barriers. There, they can be generated under the influence of microorganisms. In this issue of *Immunity*, Dutzan et al. (2017) report an important role for mechanical-damage-induced factors in specifically maintaining a Th17 cell population at the oral mucosa.

Epithelia are prime locations at risk to microbial invasion, occupied by a variety of microorganisms that make contributions to the health of the tissue as well as the host. Immune networks at epithelia balance tolerance, keep potential pathogens at bay, enable moderate immunity when minor breaches occur, and initiate comprehensive responses when encountering danger. Due to the large area it constitutes and the varied composition of its microorganisms, the intestine has attracted the most scrutiny and has been used to study and define these immune networks. Subsequently, attention was focused on the skin and lungs, with the realization that, similar to the intestine, these tissues are under the influence of micro-organisms as well. It became apparent that not just the microbial players differ between barrier organs, but that immune networks and cellular compositions vary as well. For example, the mouse skin and small intestine contain a specialized population of lymphocytes, the intraepithelial lymphocytes, that is not present in the lung or the colon and are not able to secrete IL-17 (Li et al., 2011).

Immune networks are tuned to tissue-specific needs. IL-17-producing lymphocytes present at all barrier sites are critical for protection against extracellular bacteria and fungi. They consist of those expressing a T cell receptor (TCR) formed with α and β chains (Th17 cells), such as