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Impaired Leukocyte Trafficking and Skin Inflammatory Responses in Hamsters Lacking a Functional Circadian System

Brian J. Prendergast^{1,2}, Erin J. Cable¹, Priyesh N. Patel¹, Leah M. Pyter¹, Kenneth G. Onishi¹, Tyler J. Stevenson¹, Norman F. Ruby³, and Sean P. Bradley¹

¹Department of Psychology, University of Chicago, Chicago, IL 60637

²Committee on Neurobiology, University of Chicago, Chicago, IL 60637

³Department of Biological Sciences, Stanford University, Palo Alto, CA 94305

Abstract

The immune system is under strong circadian control, and circadian desynchrony is a risk factor for metabolic disorders, inflammatory responses and cancer. Signaling pathways that maintain circadian rhythms (CRs) in immune function *in vivo*, and the mechanisms by which circadian desynchrony impairs immune function, remain to be fully-identified. These experiments tested the hypothesis that the hypothalamic circadian pacemaker in the suprachiasmatic nucleus (SCN) drives CRs in the immune system, using a non-invasive model of SCN circadian arrhythmia. Robust CRs in blood leukocyte trafficking, with a peak during the early light phase (ZT4) and nadir in the early dark phase (ZT18), were absent in arrhythmic hamsters, as were CRs in spleen clock gene (*per1*, *bmal1*) expression, indicating that a functional pacemaker in the SCN is required for the generation of CRs in leukocyte trafficking and for driving peripheral clocks in secondary lymphoid organs. Pinealectomy was without effect on CRs in leukocyte trafficking, but abolished CRs in spleen clock gene expression, indicating that nocturnal melatonin secretion is necessary for communicating circadian time information to the spleen. CRs in trafficking of antigen presenting cells (CD11c⁺ dendritic cells) in the skin were abolished, and antigen-specific delayed-type hypersensitivity skin inflammatory responses were markedly impaired in arrhythmic hamsters. The SCN drives robust CRs in leukocyte trafficking and lymphoid clock gene expression; the latter of which is not expressed in the absence of melatonin. Robust entrainment of the circadian pacemaker provides a signal critical to diurnal rhythms in immunosurveillance and optimal memory T-cell dependent immune responses.

Keywords

leukocyte trafficking; delayed-type hypersensitivity; entrainment; SCN; inflammation

Introduction

In periodic environments, circadian clocks permit synchrony of the internal milieu and anticipation of predictable changes in the environment (Pittendrigh, 1960; Bronson, 1989). Daily temporal organization of physiology and behavior is provided by entrainment of the circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) to the environmental light:dark cycle. The mammalian circadian system is composed of a network of hierarchically-interacting tissue-specific circadian clocks (Baggs et al., 2009). In order to

generate coherent physiological rhythms, the phases of these clocks are regularly reset by the output of the master circadian pacemaker in the SCN (Schibler, 2007). Perhaps the most conspicuous circadian rhythm (CR) driven by the SCN is the activity/rest cycle, but scores of physiological processes occur in a circadian manner, including endocrine function, cellular and organismal metabolism, body temperature, and countless behaviors (Takahashi et al., 2008).

Immune function is under strong circadian control. Virtually all immunological variables investigated to date exhibit circadian cycles. CRs are evident in circulating leukocyte numbers and phenotypes, lymphocyte metabolism and function, and cytokine production *in vitro* and *in vivo* (Esquifino et al., 2007). Brain-immune interactions that govern the expression of infection-induced sickness behaviors fluctuate across the circadian cycle (Arjona and Sarkar, 2005; Arjona and Sarkar, 2006; Guan et al., 2005; Marpegan et al., 2005), and mortality from sepsis varies markedly with time-of-day (Franklin et al., 2003; Franklin et al., 2007; Liu et al., 2006; Marpegan et al., 2009; Morrow and Opp, 2005; Weil et al., 2009). Furthermore, immunocompetence varies with the stability of the circadian system: extensive nighttime shift work or transmeridian travel induces chronic circadian disruption, leading to higher rates of various cancers (Pukkala et al., 2002; Reynolds et al., 2002; Schernhammer et al., 2001; Schernhammer et al., 2003), and stably-entrained behavioral and endocrine rhythms predict improved survival time in cancer patients (Mormont et al., 2000; Sephton et al., 2000). Mice with disrupted circadian phenotypes exhibit diverse immunological disorders, including enhanced tumorigenesis (Fu et al., 2002), disrupted lymphoid development (Kurebayashi et al., 2000; Sun et al., 2000), impaired T cell function and autoimmune disease (Seimiya et al., 2004), and exacerbated innate inflammatory responses (Castanon-Cervantes et al., 2010).

One major obstacle to addressing the functional significance of the circadian system in immunity has been the lack of an adequate and appropriate model system. SCN ablation reliably eliminates rhythms, but also damages substantial adjacent hypothalamic tissue, and causes nonspecific production of stress hormones (Bittman et al., 1991; Buijs et al., 1993), along with glial scars and CNS immune responses that continue for months after the insult (Logan et al., 1992; Silver and Miller, 2004). Constant light (LL) can induce arrhythmia, but such effects are transient and chronically elevate glucocorticoid secretion (Daan and Pittendrigh, 1975; Eastman and Rechtschaffen, 1983; Welberg et al., 2006). Combinations of clock gene knockouts eliminate CRs (Reppert and Weaver, 2002), but clock genes are present in all tissues, and are pleiotropic in function (Baggs et al., 2009; Greenspan, 2001; Meyer-Bernstein and Sehgal, 2001). The pleiotropy issue is of major logical importance for understanding reports of disease states in clock gene knockout mice: such effects may be due to circadian disruption (centrally or at tissue level), but may instead be due to direct roles of clock genes in cellular metabolic processes (Baggs et al., 2009; Bur et al., 2009; Kosaka and Bass, 2007; Male et al., 2012). In a single relevant lesion study, diurnal rhythms in blood leukocytes were moderately dampened (not abolished) and growth rates of implanted tumors were accelerated in mice with complete bilateral lesions of the SCN, suggesting that in the absence of circadian organization mechanisms inhibiting tumor growth are impaired (Filipski et al., 2003).

Here we use a model of chronic SCN arrhythmia generated noninvasively, thereby avoiding the pitfalls and confounds of CNS lesions, bright LL, and genetic mutations (Grone et al., 2011; Ruby et al., 2009). CRs in sleep/wake, body temperature, melatonin secretion and locomotor activity of hamsters can be eliminated within a few days in Siberian hamsters by light treatments administered once (see *Materials and Methods*). This noninvasive method for permanently eliminating CRs has the distinct advantage of allowing animals to remain undisturbed in the presence of a standard light:dark cycle and was used to investigate

functional consequences of complete circadian desynchrony on multiple aspects of immune function.

Materials and Methods

Animals

Siberian hamsters were born and raised in a 15L:9D (lights off at 18:00 h CST) breeding colony maintained at the University of Chicago. Hamsters were housed in polypropylene cages, with food (Harlan, Teklad) and filtered tap water provided *ad libitum*; cotton nesting material was also available in the cage. Ambient temperature and relative humidity were held constant at $19 \pm 2^\circ\text{C}$ and $53 \pm 10\%$, respectively. All procedures conformed to the USDA Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Experiment 1. Effects of circadian and ultradian arrhythmia on blood leukocyte trafficking

Male and female hamsters (3-5 months of age; $n=60$; 24 females, 36 males) were single-housed and transferred to 16L:8D (lights off at 18:00) for 4 weeks prior to the implementation of a circadian disrupting phase-shift (DPS) protocol (Ruby et al., 2004; see *DPS Procedure*). A group of control hamsters ($n=8$) was subjected to a sham-DPS procedure. CRs in locomotor activity were monitored over 10 consecutive days in all hamsters 1-3 months after the phase-shift was administered; this allowed identification of the presence/absence of significant CRs, and measurement of quantitative aspects of the CR waveform (see *Locomotor activity monitoring*).

Circadian leukocyte trafficking—Rhythmic aspects of blood leukocyte trafficking were assessed in 25 μl blood samples collected at 4 time points (ZT4, ZT11, ZT18 and ZT23) across the circadian cycle, over the course of 3 successive days, according to methods described elsewhere (Prendergast et al., 2003).

Stress-induced leukocyte trafficking—To elicit stress responses, hamsters (ENTR, $n=8$; ARR, $n=8$) were placed in restraint stress tubes (RST; dimensions; Bilbo et al., 2002) at ZT3 (3 h after light onset); hamsters remained in the RST for 2 h, thus the midpoint of the stressor occurred at ZT4. Control (non-stressed) hamsters remained in their home cages in a separate room. Blood (150 μl) was obtained from RS hamsters via the retroorbital sinus under isoflurane anesthesia prior to the onset of the stress/control procedure (baseline), and again 15, 60 and 120 min later; blood was obtained from controls at baseline and again 120 min later. Following each blood draw, hamsters were administered 0.2 ml warm sterile physiological saline (s.c.) to facilitate rehydration. Leukocyte concentrations were determined in a 25 μl aliquot of heparinized whole blood (see *Blood Leukocyte Quantification*).

Experiment 2. Effects of circadian arrhythmia on leukocyte trafficking and lymphoid clock gene expression under constant conditions

Female hamsters ($n=93$) were subjected to the DPS procedure; 25 (27%) reentrained to the shifted photocycle, whereas 56 (60%) became behaviorally-arrhythmic; the remainder exhibited free-running locomotor activity and were excluded from subsequent investigation. From this population, 24 ENTR and 24 ARR hamsters were subjected to an Aschoff Type II manipulation (Aschoff, 1965), which eliminates masking effects of the light-dark cycle by allowing rhythms to persist in constant conditions (continuous darkness; DD) for >2 cycles prior to sample collection. At the onset of darkness (ZT16), ENTR and ARR hamsters were transferred from 16L:8D to DD. A dim (1 lux) red light remained on at all times in DD. Retroorbital blood (25 μl) was obtained for blood leukocyte determination, and hamsters

were killed by rapid decapitation at projected ZT17 (49 h after the onset of DD) or projected ZT1 (58 h after the onset of DD). Spleens were rapidly dissected (<2 min) onto dry ice and stored at -80°C until RNA extraction.

Experiment 3. Effects of pinealectomy on blood leukocyte trafficking and lymphoid clock gene expression

Adult female hamsters (n=36) were pinealectomized (PINx) or sham-pinealectomized according to methods described by Carter and Goldman (1983; see *Pinealectomy Procedure*). Six weeks after surgery, retroorbital blood was collected at projected ZT1 and ZT17 using an Aschoff Type II design, as described above. Blood samples were obtained under isoflurane anesthesia in a pseudo-random order and leukocytes were enumerated from a 25 µl sample of whole heparinized blood. Hamsters were killed by rapid decapitation, and spleens were rapidly dissected as described for Experiment 2.

Experiment 4. Effects of circadian arrhythmia on DTH reactions

A subset of hamsters from *Experiment 1* (n=35) remained housed in 16L:8D for two weeks after blood collections were completed. In these hamsters (ENTR, n=15; ARR, n=20), delayed-type hypersensitivity skin inflammatory responses were induced by application of the antigen, 2,4-dinitro-1-fluorobenzene (DNFB; Sigma), to the pinnae of each hamster after initial DNFB sensitization. Ear inflammatory responses were monitored for 7 days following final treatment (see *DTH Procedure*).

Skin dendritic cells were quantified in a separate group of hamsters from Experiment 1 (ENTR, n=16; ARR, n=16). Under isoflurane anesthesia, skin samples from the dorsum were obtained using a sterile 4 mm punch biopsy tool, via methods described in detail elsewhere (Kinsey et al., 2003). Tissue biopsies were rinsed in sterile PBS and ground against a 40 µm cell strainer continuously for 3 minutes. Cellular exudate was strained again at 40 µm, rinsed and centrifuged (800 × g, 10 min) 2× in PBS, resuspended in PBS, and fixed with 1.5% paraformaldehyde. Before fixation, the concentration and viability of cell suspensions were assessed using trypan blue exclusion. Samples were stored at 4°C until dendritic cells were quantified via flow cytometry (see *Flow Cytometry*).

General Methods

Disruptive phase shift (DPS) procedure—The disruptive phase shift (DPS) procedure destabilizes the hamster circadian pacemaker via treatment with phase-resetting light stimuli (Ruby et al., 2004). After 4 weeks in a 16L:8D photoperiod, a 2 h light pulse was administered during the 5th through 7th h of the dark phase. On the next day, the 16L:8D photocycle was phase-delayed by 3 h, via extension of the light phase (new lights off: 21:00 h). The DPS protocol typically renders ~50% of hamsters permanently circadian arrhythmic (“ARR”; Ruby et al., 2004). Control hamsters were subjected to the 3 h phase-delay but did not receive the 2 h light pulse on the preceding night (sham-DPS); 75-90% of control hamsters re-entrain to the new photocycle (“ENTR”; Ruby et al., 2004; Prendergast et al., 2012). CRs in locomotor activity were monitored in all hamsters 1-3 months after the phase-shift was administered (cf. Ruby et al., 2009; see *Locomotor Activity Monitoring*).

Control hamsters received the 3 hour phase-delay but not the 2 h light pulse (sham-DPS). In all measures of immune function, circadian-arrhythmic hamsters that received the DPS treatment did not differ from those that received the sham-DPS treatment (blood leukocytes: $F_{1,31}=0.06$, $P>0.80$; DTH: $F_{1,31}=1.32$, $P>0.20$) and were collapsed into a single circadian arrhythmic (ARR) group (n=34). Similarly, entrained hamsters receiving the DPS treatment and those receiving the sham-DPS exhibited comparable blood leukocyte concentrations

($F_{1,27}=1.54$, $P>0.20$) and DTH reactions ($F_{1,28}<0.01$, $P>0.90$) and were combined into a single circadian entrained (ENTR) treatment group ($n=30$).

Locomotor activity monitoring—In all experiments, locomotor activity data were collected in the home cage for a minimum of 10 consecutive days with passive infrared motion detectors (Coral Plus, Visonic, Bloomfield, CT) positioned 22 cm above the cage floor. Motion detectors registered activity when 3 of 27 zones were crossed. Activity triggered closure of an electronic relay, recorded by a computer running ClockLab software (Actimetrics, Evanston, IL). Cumulative activity counts were collected at 6 min intervals for quantitative analyses of CRs.

Circadian waveform analyses—The presence/absence of CRs was determined using criteria identical to those described in prior reports of DPS-induced arrhythmia in this species (Ruby et al., 2004; Ruby et al., 2009). χ^2 periodogram analyses (ClockLab; Actimetrics) were performed on 10-day blocks of activity data. Peaks in the χ^2 periodogram were considered statistically significant if they exceeded the 99.9% confidence interval limit ($P<0.001$). Hamsters were considered arrhythmic (ARR) if there were no significant peaks in the periodogram in the circadian range, activity was distributed throughout the LD cycle, and daily rhythm onsets and offsets could not be identified via visual inspection of the periodogram (Ruby et al., 2009). Hamsters with significant circadian activity in the χ^2 periodogram were considered entrained (ENTR). Supplemental analyses after completion of χ^2 periodogram analyses were adopted as recommended by Refinetti et al. (2007); to this end, the cosinor periodogram (Bingham et al., 1982), a reliable curve-fitting tool to quantify rhythm parameters, was used (Refinetti et al., 2007). Cosinor analyses were used to calculate the mesor and amplitude of the best-fit cosinor waveform in the circadian range (22–26 h). Relative amplitude of the CR was calculated as the quotient of CR amplitude divided by CR mesor (Prendergast et al., 2012).

RNA extraction, cDNA synthesis and quantitative PCR—*per1* (period 1) and *bmal1* (brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like) mRNA expression was measured in spleen samples via qPCR. Total RNA was extracted using RNeasy (Qiagen) according to the kit instructions. Extracted RNA was suspended in 30 μ l RNase-free water and RNA concentration and quality for each sample were assessed using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was created via RT of 2 μ g RNA using MMLV RT enzyme (Invitrogen, Carlsbad, CA, USA).

In Experiment 2, primers and probes for the mRNAs of interest were designed using PrimerExpress based on Siberian hamster-specific sequences for these 3 genes published in Genbank: *per1* (AY316535), *per2* (EU AY316536) and *bmal1* (AY316534). Primers and probes were synthesized as follows, with probes labeled with 6-FAM and MGB (non-fluorescent 5'-AACTGCTTTTCTATTGCAGACAATG-3', *per2* probe 5'-AGCCACCAAGTGAGGAT-3' *bmal1* forward 5'-GGCAGCGATGGCTGTCA-3', *bmal1* reverse 5'-TCCACCCAGGCCTGCAT-3', *bmal1* probe 5'-CATGAGCCTATTGGAAGC-3'. A TaqMan 18S Ribosomal RNA primer and probe set (labeled with VIC; Applied Biosystems) was used as the control gene for quantification. Amplification was performed on an ABI 7900HT Sequencing Detection System by using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to relative standard curve consisting of serial dilutions of pooled *P. sungorus* hypothalamic cDNA (1:1, 1:10, 1:100, 1:1000, 1:10,000) followed by normalization to 18S rRNA gene expression; 6 samples were excluded due to low 18S rRNA (ENTR: ZT13, $n=2$; ARR: ZT1, $n=2$; ZT13, $n=2$).

In Experiment 3, primers for *per1* and *bmal1* were identical to those used in Experiment 2. Reporter probes were not required because qPCR was performed using iQ Sybr Green Supermix (BioRad, Hercules, CA USA). qPCR reactions used an initial denaturation at 95°C for 30 s, followed by 39 cycles of 95°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. A melting curve analysis was added to determine the quality and specificity of each reaction. Quantification of mRNA expression levels was accomplished with iQ Sybr Green Supermix (BioRad). PCR Miner (Zhao & Fernald, 2005) was used to calculate reaction efficiencies (E) and cycle thresholds (CTs). The expression of each target gene of interest was calculated relative to *gapdh* using $2^{-(\Delta\Delta Ct)}$. Four samples were identified as outliers (± 2 SD from mean) and were excluded from subsequent analyses.

Blood collection—Blood (270 μ l) was obtained under light anesthesia from the right retro-orbital sinus using heparinized Natelson collection tubes. A dim (1 lux) red light was used to facilitate dark-phase (ZT18, ZT23) collections. Blood collections were performed in pseudo-random order, in a room separate from the general animal colonies, and following the procedure, hamsters were isolated from the colony until all collections were completed. Animal handling during the blood collection was kept to a minimum (<1 min). Blood samples were deposited into heparinized (50 units) microcentrifuge tubes, mixed gently, and a 25 μ l aliquot of whole heparinized blood was removed for leukocyte determination.

Leukocyte redeployment out of the blood at each of the 3 post-peak sampling intervals (ZTx, where $x = 11, 18$ or 23) was calculated for each individual hamster as a proportion of its baseline (peak; ZT4) blood leukocyte concentration according to methods described by (Dhabhar and McEwen, 1997):

$$\% \text{redeployment} = (\text{ZT4 leukocyte number} - \text{ZTx leukocyte number}) / (\text{ZT4 leukocyte number})$$

Blood Leukocyte Quantification—Leukocyte counts from whole blood were obtained via hemolysis with 3% acetic acid at a 1:20 dilution, and enumeration in duplicate on a hemacytometer at 400 $\times$ magnification by an experimenter blind to the hamster's ultradian and circadian phenotype. Lysed samples were kept at room temperature for < 3 h before leukocyte counts were determined. Although distinct leukocyte subtypes are not identifiable with this method, this procedure reliably identifies environmentally-induced (e.g., photoperiod, stress) changes in total leukocyte number in this, and other, rodent species. In several studies of Siberian hamster leukocytopoiesis, total leukocyte counts correlates positively with photoperiodic changes in specific leukocyte subtypes, including total lymphocytes, T-cells, and NK cells (Bilbo et al., 2002; Wen et al., 2007). Circadian leukocyte trafficking requires clock-driven leukocyte migration out of the circulation to skin, organs, and lymph nodes. The measure thus provides a rapid and omnibus indicator of effects of circadian rhythmicity on the capacity for immunosurveillance in the blood. Applied longitudinally, this measure provides an accurate indicator of leukocyte trafficking (Dhabhar et al., 1995).

Pinealectomy Procedure—Anesthetized hamsters were immobilized in a stereotaxic apparatus and a midline skin incision exposed the skull from bregma to approximately 3 mm caudal to lambda. A trephine bit was centered on lambda used to remove a portion of the skull. The pineal gland was then removed with microdissecting forceps. PINx was verified via direct histological examination of the excised tissue under a dissecting microscope. Gelfoam was inserted into the wound site, and the skin closed with wound clips. Topical antibiotic ointment was applied externally. Hamsters received buprenorphine analgesic (0.01

mg/kg, sc) 2×/day for 2 days after surgery. The sham-PINx and PINx procedures were similar, except the pineal gland was not removed.

DTH Procedure—DTH skin inflammatory responses were induced by application of the antigen, 2,4-dinitro-1-fluorobenzene (DNFB; Sigma), to the pinna of each hamster after initial immunization (“sensitization”) by application of DNFB to the dorsum (Bilbo et al., 2002). Sensitization was induced and DTH elicited as follows: on all hamsters (DNFB naïve) were anesthetized with isoflurane vapor, and an area of 2 × 3 cm was shaved on the dorsum. Twenty-five μ l of DNFB [0.5% (wt/vol) in 4:1, acetone/olive oil vehicle] was applied to the dorsal skin on 2 consecutive days. Six days later, baseline thickness of both pinnae was measured under light anesthesia using a constant-loading dial micrometer (± 0.01 mm; Mitutoyo, Tokyo). Immediately after baseline pinna measurements were obtained, the DTH reaction was elicited by applying 20 μ l of DNFB [0.2% (wt/vol) in 4:1, acetone/olive oil] to the dorsal skin of the right pinna. Left pinnae were treated with vehicle and served as a control treatment. Pinna thicknesses were measured every day for the next 7 days. All measurements were performed by the same experimenter who was blind to ultradian/circadian phenotype. Pinna thickness values obtained on each day following challenge were expressed as a percentage of baseline thickness for statistical calculations. All DNFB treatments and pinna measurements were performed between 14:00–15:00 h (ZT12–13), and all measurements were made on the same relative region of the pinna. This regimen of sensitization and challenge uses concentrations of DNFB that differ from those used in rats (Dhabhar and McEwen, 1999), but have been optimized for use in Siberian hamsters (Bilbo et al., 2002; Prendergast and Pyter, 2009). DTH reactions require antigen capture, drainage of antigen-presenting cells from the skin to the lymphatic system, antigen presentation, clonal T cell expansion and, following antigen challenge, a rapid deployment of leukocytes out of the blood and infiltration into the epidermis and dermis (Dhabhar, 2000). DTH is a standard *in vivo* measure of T cell mediated immunity (Turk, 1980).

Flow Cytometry—Appropriate dilutions of each monoclonal antibody (Ab) were established to use saturating antibody concentrations. Extracted cells from the entire skin biopsy were centrifuged at $800 \times g$ for 10 min, resuspended PBS with 0.5% BSA, and incubated with Ab (at appropriate concentrations as determined by titration, see below) for 30 min at 20°C. Stained cells were then washed 2× in PBS, and resuspended in 200 μ l PBS/BSA plus 50 μ l counting beads. Negative-controls and single-stained controls were included for use in gating and compensation, using FlowJo version 9.5.4 (Tree Star Inc., Palo Alto, CA). All samples were analyzed using the same gates. For each molecule studied, all samples were analyzed with the same conditions of voltage on the cytometer.

Lymphocytes and granulocytes were identified based on forward and side scatter properties. Leukocytes were identified as CD45⁺ (using FITC-conjugated anti-mouse-CD45; clone 30-F11, 1:200; BioLegend, San Diego, CA). Dendritic cells (DCs) were identified as CD11c⁺ (using PE-conjugated, anti-mouse-CD11c; clone BU15, 1:5; BioLegend). CD11c is a standard marker for DCs (Brocke, 1997; Metlay et al., 1990); mature dendritic cells were identified as CD45⁺/CD11⁺ lymphocytes with high SSC, indicative of extensive dendritic outgrowth (Wang et al., 2009). Absolute numbers of cells per μ l of skin extract were determined via the addition of 50 μ l polystyrene microbeads (3.4 μ m diameter, 43500 beads; Spherotech, Lake Forest, IL) to each sample. Following cytometry, the formula: (# CD11c⁺ events/# microbead events) $\times$ (217.5 beads/ μ l initial sample) was used to calculate the number of DCs per μ l of sample (Ben-Eliyahu et al., 2000; Neeman et al., 2012). The quotient of total DCs divided by the area skin obtained by the 4 mm diameter biopsy tool (12.57 mm²) provided a measure of skin DC density. Due to unequal variances, data for all groups were log10 transformed for statistical evaluation.

Plasma cortisol—Cortisol was measured in plasma (diluted 1:40) in a single EIA (Correlate-EIA; Assay Designs, Ann Arbor, MI, USA) that has been validated for this species (Demas et al., 2004) according to the manufacturer's instructions. The cortisol EIA had a sensitivity of < 57 pg/ml, an intra-assay CV of <10.5% and an inter-assay CV of <8.6%.

Statistical analyses—Cosinor analyses were performed using software written by R. Refinetti (available at <http://www.circadian.org/software.html>). Repeated measures ANOVAs were used to evaluate the effects of circadian phenotype on diurnal rhythms in blood leukocytes and on the evolution of the DTH reaction. ANOVAs were used to evaluate the effects of circadian phenotype, circadian phase and surgical treatment on blood leukocyte values, spleen clock gene expression, and skin dendritic cell concentrations. *A priori* planned pairwise comparisons were performed using *t* tests. Differences were only considered significant if *P* < 0.05.

Results

Circadian arrhythmia abolishes light-entrained rhythms in leukocyte trafficking

Circadian rhythms in behavior—Hamsters in a 16L:8D photocycle were subjected to a disruptive phase shift (DPS) treatment, consisting of a 2 h pulse of light at night, followed by a 3 h phase-delay of the photocycle (see *Materials and Methods*), and home-cage locomotor activity of each hamster was monitored for 10 days beginning >1 month after DPS treatment. Among DPS-treated hamsters, 52% (31/60) became circadian arrhythmic (ARR), 40% (24/60) continued to express entrained CRs (ENTR), and 8% (5/60) exhibited free-running locomotor activity rhythms (Fig. S1). Free-running hamsters were excluded from subsequent analyses. Among hamsters subjected to a control treatment (a 3 h phase delay without the 2 h light pulse), 75% (6/8) re-entrained normally, and 25% (2/8) became arrhythmic (cf. Ruby et al., 2004).

CRs in blood leukocyte trafficking—Rhythmic aspects of blood leukocyte trafficking were assessed in blood samples from ENTR (n=29) and ARR (n=33) hamsters collected at 4 time points across the circadian cycle: Zeitgeber time 4 (ZT4; 4 h after the onset of the light phase), ZT11, ZT18 and ZT23). Circadian phenotype significantly affected the daily pattern of blood leukocyte concentrations ($F_{3,180}=11.1$, $P<0.001$; Fig. 1A). Among ENTR hamsters, blood leukocytes peaked early in the light phase (ZT4) and reached a nadir shortly after the onset of darkness (ZT18). The diurnal waveform of blood leukocytes was markedly dampened among ARR relative to ENTR hamsters ($P<0.001$). Leukocyte concentrations were significantly lower in ENTR relative to ARR hamsters at ZT18 ($t_{60}=9.22$, $P<0.001$) and ZT23 ($t_{60}=6.54$, $P<0.001$).

Circadian phenotype also affected the pattern of leukocyte redeployment out of the blood ($F_{2,120}=9.85$, $P<0.001$; Fig. 1B). When calculated relative to peak (ZT4) blood leukocyte values, significant leukocyte redeployment was evident in ENTR ($F_{2,56}=21.4$, $P<0.001$) but not in ARR ($F_{2,64}=2.60$, $P>0.05$) hamsters. Leukocytes deployment out of the blood was greater in ENTR relative to ARR hamsters at ZT18 ($t_{60}=-7.63$, $P<0.001$) and ZT23 ($t_{60}=-5.10$, $P<0.001$; Fig. 1B).

Stress-induced blood leukocyte trafficking—To evaluate the effect of circadian arrhythmia on stress-induced leukocyte trafficking, blood leukocyte concentrations were measured in ARR (n=8) and ENTR (n=8) hamsters prior to, and during, a 2 h restraint stress (RS) manipulation from ZT3 - ZT5. ENTR and ARR hamsters exhibited comparable leukocyte trafficking responses to RS ($F_{1,12}=0.01$, $P>0.90$; Fig. 1C). In both phenotypes, RS

caused a significant redeployment of leukocytes out of the blood (+60 min: $P<0.01$; +120 min: $P<0.01$ vs. pre-stress values). Leukocyte trafficking responses to RS were comparable in ENTR and ARR hamsters at all time points post-stress ($P>0.20$, all comparisons; Fig. 1C).

In hamsters (ARR, $n=6$; ENTR, $n=7$) not exposed to RS, plasma cortisol concentrations at ZT8 (around the cortisol nadir, at the midpoint of the light phase) did not differ between circadian phenotypes (ENTR: 85.0 ± 5.8 ng/ml; ARR: 82.5 ± 6.2 ; mean $\pm$ SEM; $t_{11}=0.3$, $P>0.70$).

Circadian arrhythmia eliminates endogenous rhythms in leukocyte trafficking and lymphoid clock gene expression

CRs in blood leukocyte trafficking—The next experiment examined whether CRs in blood leukocytes reflect endogenous rhythms in leukocyte trafficking, and whether circadian oscillations in clock gene expression in peripheral lymphoid tissues require a functional SCN. ENTR ($n=24$) and ARR ($n=24$) hamsters were transferred to constant darkness; blood and spleen samples were collected after >48 h in constant darkness (DD) at projected ZT1 and ZT17 (Aschoff et al., 1965). Circadian phenotype significantly affected blood leukocyte values in DD ($F_{1,44}=13.2$, $P<0.001$; Fig. 2A): CRs in blood leukocyte trafficking were sustained in DD among ENTR hamsters, with concentrations approximately 2-fold higher at ZT1 relative to ZT17 ($t_{22}=4.92$, $P<0.001$). In contrast, among ARR hamsters leukocyte concentrations were comparable at ZT1 and ZT17 ($t_{22}=-0.635$, $P>0.50$; Fig. 2A).

Spleen clock gene expression—Circadian clock gene (*per1*, *bmal1*) expression in the spleen of ENTR and ARR hamsters was also measured after 48 h in DD using qPCR. Among ENTR hamsters, *per1* mRNA was significantly lower at ZT1 relative to ZT17 ($t_{18}=-2.82$, $P=0.01$), and *bmal1* mRNA was significantly higher at ZT1 relative to ZT17 ($t_{18}=2.02$, $P<0.05$; Fig. 2B). In contrast, among ARR hamsters ZT1/ZT17 differences in *per1* ($t_{16}=1.14$, $P>0.20$) and in *bmal1* ($t_{16}=1.05$, $P>0.30$) were not statistically significant (Fig. 2B).

Total daily mRNA expression (collapsed across ZTs) was comparable in ENTR and ARR hamsters (*per1*: $F_{1,36}=2.15$, $P>0.10$; *bmal1*: $F_{1,36}=0.06$, $P>0.80$; not illustrated), but circadian phenotype altered the ratio of *per1:bmal1* mRNA in the spleen ($F_{1,34}=21.3$, $P<0.001$; Fig. 2C). ENTR hamsters exhibited robust circadian variation in *per1:bmal1* (*per1:bmal1*: $t_{18}=-4.86$, $P<0.001$), whereas in ARR hamsters *per1:bmal1* values remained low and did not vary over the subjective circadian cycle ($t_{16}=1.03$, $P>0.30$; Fig. 2C).

Melatonin imparts circadian time information onto peripheral oscillators in lymphoid tissues

CRs in blood leukocyte trafficking—Pineal melatonin (MEL) secretion is a high-amplitude circadian endocrine signal and melatonin signaling exerts diverse immunomodulatory effects (Carrillo-Vico et al., 2005). Thus, the next experiment examined whether nocturnal MEL secretion is necessary to maintain CRs in blood leukocyte trafficking and in spleen clock gene expression. ENTR hamsters were pinealectomized (PINx; $n=16$) or sham-operated ($n=20$), and six weeks later blood and spleens were collected >48 h after transfer to DD. CRs in blood leukocytes were not affected by PINx ($F_{1,32}=0.72$, $P>0.70$; Fig. 3A): circadian leukocyte trafficking was evident in both groups, with greater blood leukocyte concentrations at ZT1 relative to ZT17 (sham-PINx: $t_{18}=6.44$, $P<0.001$; PINx: $t_{14}=3.69$, $P<0.001$; Fig. 3A).

Spleen clock gene expression

In control hamsters spleen *per1* expression was higher at ZT17 relative to ZT1 ($t_{15}=-1.79$, $P<0.05$, one tailed), and *bmal1* expression was higher at ZT1 relative to ZT17 ($t_{18}=2.35$, $P<0.05$; Fig. 3B); PINx eliminated ZT1/ZT17 differences in *per1* and *bmal1* expression in DD ($P>0.90$ and $P>0.70$, respectively). In sham-PINx hamsters, the *per1:bmal1* mRNA ratio in spleen changed markedly with circadian time: *per1:bmal1* was higher at ZT17 relative to ZT1 ($t_{15}=-2.64$, $P<0.05$; Fig. 3C). Circadian changes in *per1:bmal1* were eliminated by PINx ($t_{12}=-0.57$, $P>0.50$; Fig. 3C).

Total *per1* mRNA expression (collapsed across ZTs) was significantly greater in PINx relative to sham-PINx hamsters ($F_{1,29}=8.87$, $P>0.01$; not illustrated); omnibus *bmal1* expression was not affected by surgical treatment ($F_{1,32}=0.22$, $P>0.60$; not illustrated).

Effects of circadian arrhythmia on DTH reactions and skin leukocyte trafficking

DTH reactions—Delayed-type skin hypersensitivity (DTH) reactions were elicited in a subset of ENTR ($n=15$) and ARR ($n=20$) hamsters from Expt 1. DTH was induced by application of the antigen, 2,4-dinitro-1-fluorobenzene (DNFB; Sigma), to the pinna of each hamster after initial immunization (sensitization, by application of DNFB to the dorsum); sensitization and challenge both occurred between ZT12 and ZT13. Circadian phenotype significantly altered the magnitude and waveform of the DTH inflammatory reaction over several days ($F_{7,231}=6.53$, $P<0.001$): ear thicknesses were greater in ENTR relative to ARR hamsters on *day* +2 ($P<0.001$) and *day* +3 ($P<0.005$) after challenge (Fig. 4A).

Skin dendritic cells—To examine if impaired DTH reactions in ARR hamsters are accompanied by changes in mechanisms that participate in antigen presentation, activated skin dendritic cells (CD45⁺/CD11c⁺/SSC^{high}) were quantified in ARR ($n=8$) and ENTR hamsters ($n=8$) from punch biopsies obtained between ZT2-ZT3 and between ZT17-ZT18 (hereafter, 'ZT3' and 'ZT18'; $n=8$ /phenotype/time). In ENTR hamsters, CD45⁺/CD11c⁺ skin dendritic cells were 3-fold higher at ZT18 relative to ZT3 ($t_{14}=-2.50$, $P<0.025$; Fig. 4B); diurnal variation in skin dendritic concentrations were not evident in ARR hamsters ($t_{14}=0.93$, $P>0.30$).

Quantitative relations between circadian amplitude and immune function

Linear regression analyses identified a significant positive correlation between amplitude of the circadian locomotor activity rhythm and the amplitude of the CR in blood leukocytes ($\beta=0.56$, $R^2=0.32$, $P<0.001$). CR amplitude also predicted the amplitude of the peak (*day* +2) DTH response ($\beta=0.46$, $R^2=0.21$, $P<0.01$; Fig. 5A, 5B).

Discussion

Disruptive phase shifts (DPS) induced behavioral arrhythmia (Fig. S1), abolished circadian control of leukocyte trafficking (Fig. 1), rendered peripheral clocks in lymphoid tissues arrhythmic (Fig. 2), and impaired organismal-level antigen-specific immune responses (Fig. 4). Critical to the interpretation of the present data is that DPS-induced arrhythmic hamsters, unlike SCN ablation models of arrhythmia, did not sustain trauma or injury to the CNS. Brain lesions result in CNS inflammation that persists for months after surgery (Norenburg, 1994; Suzuki et al., 2012), and bone remodeling and cutaneous wound healing that continues for the remainder of the lifespan (Lalani et al., 1998)— processes which permanently alter immune function and confound interpretation of measures of immunity. In contrast to lesion models, DPS-induced ARR hamsters differ from ENTR controls only in that the pacemaker in the SCN is driven to a state of near zero amplitude (Steinlechner et al., 2002); expression of core circadian clock genes in the SCN is low-amplitude and arrhythmic

(Grone et al., 2011), giving rise to profound arrhythmia in locomotor activity, sleep, and body temperature (Larkin et al., 2004; Ruby et al., 2009) and eliminating nocturnal melatonin secretion (Steinlechner et al., 2002). Circadian clock genes are increasingly recognized as important to cellular immune processes (*clock*: [Spengler et al., 2012]; *cry*: [Narasimamurthy, 2012]; *reverb*: [Gibbs et al., 2012]; *per*: [Liu et al., 2006]; *e4bp4*: [Male et al., 2012]). Unlike transgenic and mutation models of arrhythmia, however, DPS-arrhythmic hamsters possess the full complement of clock genes. Thus, the present data suggest that, among the pleiotropic effects of clock genes and their protein products, the conferral of time information *per se* is of functional significance towards organismal-level immune function.

Immune function is under strong circadian control: CRs in blood leukocytes are well-documented (see Dhabhar et al., 1994), and innate (Marpegan et al., 2009) and adaptive (Pownall et al., 1979) inflammatory responses vary over circadian time. Here we show that the functional integrity of the circadian pacemaker in the SCN is required to impart temporal organization to leukocyte trafficking and to tissue-level circadian clocks in secondary lymphoid tissues, the sites of lymphocyte activation by antigen (Mebius and Kraal, 2005). In addition, rhythmic clock gene expression in the spleen was absent in hamsters without nocturnal pineal melatonin secretion, but free-running rhythms in leukocyte trafficking persisted in the absence of melatonin. The present work also shows that the role of the circadian system in immune function goes beyond merely providing temporal phase information. In addition to losing diurnal rhythms in skin antigen (DTH) responses. Together, the data indicate that circadian organization is essential for coordination of organismal-level immune function and identify a novel neuroendocrine mechanism by which circadian time information is communicated from the central pacemaker to the peripheral immune system.

SCN arrhythmia abolishes circadian rhythms in leukocyte trafficking

In ARR hamsters, CRs in leukocyte trafficking were eliminated. Notably, this did not manifest as leukopenia, which would be indicative of immunosuppression; rather, blood leukocyte concentrations were maintained throughout the day near peak levels, with little evidence of masking effects of light. In ENTR hamsters, blood leukocytes peaked at the beginning of the rest phase (ZT4) and reached a nadir shortly after the beginning of the active phase (ZT18). In rats, blood leukocytes exhibit a similar waveform (Dhabhar et al., 1994, and circadian trafficking out of the blood is primarily accomplished by CD4⁺ T cells, B cells, NK cells and monocytes (Dhabhar et al., 1994). The present data indicate that a functional pacemaker in the SCN is required for the expression of diurnal rhythms in leukocyte trafficking; indeed, even in the presence of a full light-dark cycle CRs in blood leukocyte trafficking were absent in ARR hamsters. Other models of circadian arrhythmia have suggested the existence of non-dampening circadian oscillators in the immune system, capable of sustaining CRs in lymphopoiesis or trafficking in the absence of a functional central pacemaker: CRs in blood leukocytes persisted in rats after 8 weeks of exposure to LL (Deprés-Brummer et al., 1997), and after 11 weeks in LL, low-amplitude CRs in CD4⁺ and CD8⁺ T cells persisted, despite the loss of CRs in total lymphocytes (Deprés-Brummer et al., 1997). Chronic LL reliably eliminates behavioral and thermoregulatory CRs, but CRs in SCN activity (clock gene expression, *c-fos*) are not consistently eliminated by LL treatments (Beaulé et al., 2003; Edelstein and Amir, 1996; Nováková et al., 2011). Residual, low-amplitude CRs in the SCN, while inadequate to drive behavioral or thermoregulatory rhythms, may be of sufficient amplitude to sustain rhythms in leukocyte trafficking under chronic LL treatment.

In contrast to circadian-driven leukocyte trafficking, trafficking in response to an acute stressor was normal in ARR hamsters (Fig. 1C), indicating that the capacity for leukocyte

trafficking may be unimpaired by the loss of central rhythmicity; decrements in leukocyte trafficking in ARR hamsters are selective-- specific to trafficking responses to circadian time cues.

The SCN regulate tissue-level synchrony in clock gene expression

Lesions of the SCN eliminate CRs in clock gene expression in multiple peripheral organs (Akhtar et al., 2002; Guo et al., 2006; Sakamoto et al., 1998). The spleen is a key organ for immunological homeostasis: both innate and adaptive immune responses can be initiated and mounted in the spleen (Mebius and Kraal, 2005). In the present study, ZT1/ZT17 differences in splenic *per1* and *bmal1* expression were absent in ARR hamsters. Such an outcome may arise from either tissue-level arrhythmia or asynchronous rhythmicity between individuals. If tissue-level rhythmicity persists in individual ARR hamsters, then *per1* and *bmal1* mRNAs levels should remain in antiphase, as in ENTR hamsters (cf. Fig. 2C). In addition, if individual hamsters within a treatment group retain free-running tissue-level rhythms, but are merely desynchronized at a population level, then *per1:bmal1* variance should be considerably greater among ARR relative to ENTR hamsters across the circadian day. Neither of these outcomes was obtained. Spleen *per1:bmal1* ratios in ARR hamsters were low and invariant over circadian time in DD, and the SEM of this ratio was consistently smaller in ARR relative to ENTR hamsters (Fig. 2C). This outcome is consistent with an arrest of free-running rhythms in cellular circadian clocks in the spleen following the induction of SCN arrhythmia (Guo et al., 2006). The data corroborate previous reports in SCN-lesioned Syrian hamsters (Guo et al., 2006) and mice (Guo et al., 2005) which demonstrated peripheral arrhythmia following loss of SCN-derived time information, suggesting that the SCN is a pacemaker, rather than a phase-coordinator, of peripheral oscillators (Guo et al., 2006). Importantly, the present data demonstrate peripheral immunological arrhythmia in neurologically-intact, unoperated hamsters, permitting the inference that neither the loss of fibers of passage, nor pathological inflammatory (Norenburg, 1994; Suzuki et al., 2012) or neuroendocrine (Bartness et al., 1991; Bittman et al., 1991) sequelae common to postoperative physiology are responsible for the loss of peripheral tissue oscillations. Rather, the mere dampening of rhythms in clock gene expression in the SCN is sufficient to disengage immunological peripheral oscillators from the central pacemaker.

Circadian output to the spleen depends on nocturnal melatonin secretion

Data from ARR hamsters imply that, under conditions of normal entrainment, signals emanate from the SCN to impose temporal order on leukocyte trafficking and spleen clock gene expression. We tested the hypothesis that the high-amplitude nocturnal rhythm in pineal melatonin (MEL) secretion constituted a coupling mechanism between the central pacemaker in the SCN and circadian events in the immune system. The SCN drives nocturnal MEL secretion via a polysynaptic central-sympathetic pathway (Moore, 1996); SCN lesions abolish MEL secretion (Bittman et al., 1991), and MEL secretion is likewise absent in behaviorally-arrhythmic Siberian hamsters (Schöttner et al., 2011). PINx did not affect leukocyte trafficking (Fig. 3A), but eliminated CRs in splenic *per1* and *bmal1* mRNA expression (Fig. 3B). As in the previous experiment, *per1:bmal1* ratios were less variable in PINx relative to sham-PINx hamsters, consistent with the interpretation that the absence of nocturnal MEL secretion led to a loss of phase coherence among cells in the spleen and tissue-level arrhythmia of the splenic clock. Importantly, PINx does not alter the pattern of SCN entrainment to the external photocycle (Prendergast and Freeman, 1999); PINx hamsters lack only MEL secretion concurrent with the nocturnal active phase (Bartness et al., 1993). Specification of the molecular mechanisms by which PINx abolishes clock gene rhythms was beyond the scope of the present investigation, but MEL is sufficient to set the phase of *Rev-erba* α expression (Wagner et al., 2007), and *Rev-erba* α repression of *bmal1*

expression plays a critical role in driving CRs in macrophage function (IL-6 responses to endotoxin; [Gibbs et al., 2012]). In the absence of MEL, rhythmic inhibition of *bmal1* by *Rev-erb a* may be sufficiently diminished in ARR hamsters to give rise to tissue-specific arrhythmia.

Circadian integrity is essential for coordinating organismal-level inflammatory responses

Antigen-specific T cell responses vary markedly over the circadian day *in vivo* and *in vitro*, and functional T cell rhythms are eliminated in *Clock*^{-/-} mice, suggesting important roles for CNS and cellular clocks in T cell function (Fortier et al., 2011). The present study identified the importance of CNS-derived circadian time information in the genesis of T cell-dependent immune responses. Both ENTR and ARR hamsters exhibited skin inflammatory (DTH) reactions, but DTH was markedly reduced in ARR hamsters. This suggests that the mechanisms underlying memory T cell dependent skin inflammatory responses are impaired following loss of CNS circadian rhythmicity. This outcome stands in contrast with a report of enhanced contact hypersensitivity (CHS) responses in *Clock*^{-/-} mice (Takita et al., 2013). However, plasma corticosterone (B) concentrations are markedly reduced in *Clock*^{-/-} mice, and effects of this genotype on CHS are eliminated by B or dexamethasone treatment (Takita et al., 2013), suggesting that the immunophenotype is likely mediated by a pleiotropic effect of CLOCK on neuroendocrine function.

Transient circadian desynchrony potentiates innate inflammatory responses (Castanon-Cervantes et al., 2010), but the present data indicate that chronic circadian desynchrony inhibits adaptive inflammatory responses. Either the formation, retention or recall of immunological memory is diminished in the absence of circadian time in the internal milieu. The mediators of this immunosuppression are not yet known. The SCN regulates rhythms in corticosterone secretion (Buijs et al., 1999) and in leukocyte trafficking (Figs. 1, 2). Chronically-elevated glucocorticoids inhibit skin DTH reactions (Dhabhar and McEwen, 1997); however, the present data indicate that daytime (resting) cortisol is not elevated in ARR relative to ENTR hamsters. Skin dendritic cells (DCs) play a critical role in antigen presentation during inflammatory responses (Varona et al., 2001); and antibodies that block DC CD11c binding markedly inhibit DTH reactions (Sadhu et al., 2007). The absence of diurnal rhythms in skin DC trafficking, and in omnibus leukocyte trafficking, in ARR hamsters may decrease the efficiency of multiple stages of the DTH reaction.

Together, the present data indicate widespread impairments in immunity following the loss of central circadian rhythmicity. Understanding how the circadian pacemaker affects the immune system may afford novel insights into how interactions with the circadian environment (lighting, shift work, transmeridian travel) affect health and disease. Indeed, recent work in this species indicates that dim illumination at night (5 lux) inhibits DTH and plasma bactericidal activity (Bedrosian et al., 2011), suggesting that either CR disruption or non-circadian masking effects of light are sufficient to inhibit immune responses. Importantly, in the present study, significant relations were evident between the degree of circadian disruption and decrements in leukocyte trafficking (Fig. 5A) and DTH responses (Fig. 5B). Together, these data suggest that immunological deficits may not be solely revealed under conditions of complete circadian arrhythmia. Abundant epidemiological data support an association between non-traditional work schedules or transmeridian travel and increased risks of multiple cancers, inflammatory diseases and metabolic disorders (Erren et al., 2008; Karlsson et al., 2005). Poor circadian hygiene (Mill et al., 2004), even in the absence of arrhythmia, may lead to impairments in immune responses that require the formation of immunological memory (e.g., DTH, antibody production, autoimmunity) or precise circadian control of leukocyte trafficking (e.g., skin inflammation, wound healing).

Supplementary Figure 1 (S1)

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Figure 1. Circadian arrhythmia eliminates diurnal rhythms in blood leukocyte trafficking

(A) Mean $\pm$ SEM concentration of blood leukocytes at 4 time points over the circadian day in hamsters that were entrained to the light-dark cycle (ENTR, $n=29$) or were rendered behaviorally arrhythmic via DPS treatment (ARR, $n=33$; see *Methods*). Data are double-plotted. (B) Mean $\pm$ SEM blood leukocyte trafficking (as % ZT4 value) responses of ENTR and ARR hamsters over the circadian cycle. (C) Mean $\pm$ SEM blood leukocyte trafficking responses of ENTR ($n=8$) and ARR ($n=8$) hamsters to a 2 h restraint stress manipulation delivered from ZT3 - ZT5. *** $P<0.001$ vs. ARR value at similar time point. # $P<0.01$ vs. pre-stress value, within phenotype.

Figure 2. The SCN regulates tissue-level synchrony in splenic clock gene expression
Mean $\pm$ SEM (A) concentration of blood leukocytes, (B) spleen *per1* and *bmal1* mRNA expression, and (C) ratio of *per1*:*bmal1* mRNA of ENTR (n=24) and ARR (n=24) hamsters at projected ZT1 and ZT17, 48 h after transfer to continuous darkness (DD). **P<0.01, ***P<0.001. ###P<0.001 vs. ZT1 value.

Figure 3. Circadian output to the spleen requires melatonin secretion

Mean $\pm$ SEM (A) concentration of blood leukocytes, (B) spleen *per1* and *bmal1* mRNA expression, and (C) ratio of *per1*:*bmal1* mRNA of pinealectomized (PINx, n=16) and pineal-intact (sham-PINx, n=20) entrained (ENTR) hamsters at projected ZT1 and ZT17, 48 h after transfer to continuous darkness (DD). *P<0.05, **P<0.01).

Figure 4. Circadian arrhythmia impairs organismal-level inflammatory responses and trafficking of antigen-presenting cells

(A) Mean $\pm$ SEM delayed-type hypersensitivity (DTH) responses (pinna thickness) during the week post-antigen (DNFB) challenge of ENTR (n=15) and ARR (n=20) Siberian hamsters. DNFB sensitization, challenge, and all ear measurements were performed between ZT12 and ZT13. (B) Mean $\pm$ SEM number of dendritic cells (CD45⁺/SSC^{high}/CD11⁺) in the skin of ENTR and ARR hamsters at ZT3 and ZT18. **P<0.005, ***P<0.001 vs. ARR value on same day. #P<0.05 vs. ZT3 value, within phenotype.

Figure 5. Amplitude of the circadian waveform predicts the magnitude of leukocyte trafficking and adaptive immune responses

Linear regression between amplitude of the circadian locomotor activity waveform and (A) peak leukocyte redeployment, and (B) peak skin inflammatory responses. Both regression coefficients were statistically different from zero (leukocyte trafficking: $P < 0.001$; DTH inflammatory response: $P < 0.01$).