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Circadian Variation of the Response of T Cells to Antigen

Erin E. Fortier,*†‡ Julie Rooney,‡ Hugues Dardente,*§ Marie-Pierre Hardy,‡ Nathalie Labrecque,†‡ and Nicolas Cermakian*§

Circadian clocks regulate many important aspects of physiology, and their disturbance leads to various medical conditions. Circadian variations have been found in immune system variables, including daily rhythms in circulating WBC numbers and serum concentration of cytokines. However, control of immune functional responses by the circadian clock has remained relatively unexplored. In this study, we show that mouse lymph nodes exhibit rhythmic clock gene expression. T cells from lymph nodes collected over 24 h show a circadian variation in proliferation after stimulation via the TCR, which is blunted in Clock gene mutant mice. The tyrosine kinase ZAP70, which is just downstream of the TCR in the T cell activation pathway and crucial for T cell function, exhibits rhythmic protein expression. Lastly, mice immunized with OVA peptide-loaded dendritic cells in the day show a stronger specific T cell response than mice immunized at night. These data reveal circadian control of the Ag-specific immune response and a novel regulatory mode of T cell proliferation, and may provide clues for more efficient vaccination strategies. The Journal of Immunology, 2011, 187: 6291–6300.

Numerous physiological processes show cyclical variations with a period close to 24 h, known as circadian rhythms, even in the absence of environmental time cues (1). These rhythms are generated by circadian clocks located in various organs and cell types in the CNS (2) and in most peripheral tissues (3). Despite this distributed nature of the circadian system, a master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus receives input from the retina and coordinates rhythms in other tissues, thereby mediating their synchronization to the environmental cycles.

At the molecular level, circadian clocks consist of a set of clock genes (1, 4). The basic mechanism relies on the activation of the clock genes Period (Per) 1 and 2 and Cryptochrome (Cry) 1 and 2 by the CLOCK/BMAL1 dimer, and the negative feedback of PER and CRY proteins on CLOCK/BMAL1 activity, and consequently their own expression. CLOCK/BMAL1 also activate genes encoding nuclear receptors such as REV-ERB and ROR factors, which regulate Bmal1 gene transcription. These feedback loops result in rhythmic expression of RNAs encoded by clock genes as well as clock-controlled genes. Up to 10% of genes in a given tissue can be clock controlled, and the set of clock-controlled genes is tissue specific (5–7). Circadian clocks are present in most tissues, and, often, in organs that mediate the effects of environmental influences. Tissues that play important roles in metabolism, arousal, and stress response contain circadian oscillators.

The function of the immune system is to orchestrate a highly regulated and specific cell-mediated defense against foreign particles and pathogens. T lymphocytes are key in the adaptive response to infection. In the lymph nodes, these cells encounter and recognize, through their variable TCR, peptidic fragment of the Ag in association with self-molecules of the MHC on APCs. This recognition elicits downstream signaling that induces two crucial events, as follows: T cell proliferation, which dramatically increases the frequency of Ag-specific cells, and the differentiation into effector cells that eliminate the rapidly dividing pathogen (8, 9).

The diurnal regulation of immune processes and responses has been known for a long time (10). In rodents, susceptibility to lethal doses of the bacterial endotoxin LPS presents a diurnal rhythm (11, 12) and is exacerbated upon circadian disruption (13). Similarly, mortality following pneumococcal infection varies according to time of day (14). In humans, cases of asthma symptoms and rheumatoid arthritis occur more at certain times of the day (15, 16). However, the basis for this rhythmic regulation remains unclear. Various immune cell populations and cytokines vary over the day in the blood of humans and rodents (10, 17–21). One study presented persistent rhythms in lymphocyte counts when the SCN central clock was inactivated by constant light, leading the authors to propose the existence of immune oscillators (20).

The existence of such oscillators has been supported by the expression of clock genes in blood cells of both humans (22–24) and rodents (13, 25, 26) as well as in lymphoid organs such as bone marrow, thymus, spleen, and lymph nodes (LNs) (13, 25, 27–31). What is the functional consequence of these immune clocks?
The response of NK cells to stimulating signals has been reported to exhibit a rhythm, which depends on clock genes within these cells (18). Also, the phagocytic function of macrophages (32) and the cytokine release by these cells in response to LPS (30) present a circadian rhythm, which was proposed to depend on a clock in these cells. Many of the studies published to date have focused on the innate or non-Ag–specific immune response. Much less is known about the circadian regulation of events occurring in secondary lymphoid organs such as the LNs, and more generally of the adaptive immune response.

In this study, we show that the T cell response to a TCR trigger varies in a circadian manner. This is not based on the daily variation in the number of T cell subsets in the LN nor on the expression level of the TCR. Instead, we demonstrate a circadian variation in the protein levels of the TCR-associated kinase ZAP70. Finally, we show that the in vivo Ag-specific response of T cells is influenced by the timing of immunization.

Materials and Methods

Animals

Clock mutant mice (33) were obtained from The Jackson Laboratory and bred in house. The Clock mutant mice used in the T cell proliferation experiments had a pure C57BL/6J background, whereas those used for RNA extraction and quantitative PCR experiments had a 50:50 C57BL/6j: BALB/c background. All other experiments were done with C57BL/6 mice (Charles River Laboratories). In all cases, young adult males were entrained to a 12:12 h light:12 h dark (LD) cycle for at least 2 wk before experimentation. For RNA, cell subpopulation, and proliferation experiments, animals were placed in constant darkness (DD) after LD entrainment and sacrificed by cervical dislocation under dim red light at the indicated times on the second day of DD. For dendritic cell (DC)–OVA immunizations, mice were kept and used under LD conditions. When animals are in LD, Zeitgeber time (ZT0) is defined as the time of lights on and ZT12 is the time of lights off. When animals are in DD, circadian time (CT)12 is defined as the time of activity onset, equivalent to the time of lights off under the previous LD cycle. Thus, CT0-12 is subjective day and CT12-24 is subjective night. All procedures involving animals were carried out in accordance with guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committee of the Douglas Mental Health University Institute.

RNA extraction and quantitative PCR

On the second day in DD, superficial inguinal, brachial, and axillary LNs were collected every 6 h over 24 h at the time points indicated for each experiment and frozen on dry ice. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Reverse transcription was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using an Applied Biosystems Real-Time Cycler ABI Prism 7500 with TaqMan gene expression assays (Per2, Mm00478113_m1; Rev-erba, Mm00520708_m1; Zap70, Mm0049255_m1; Applied Biosystems), according to the manufacturer’s instructions. β-actin was used as an endogenous control (4352341E; Applied Biosystems), and the maximal value was set as 1.0.

RNase protection assay

For each clock gene studied by RNase protection assays (RPAs), the cDNA was generated from mouse RNAs by RT-PCR and cloned into pBluescript (Stratagene). The cDNAs cover the following sequences: Per1, nt 226–562 of GenBank entry NM_011065; Per2, nt 456–585 of AF036893; Cry1, nt 1394–1909 of AF156986; Dec1, nt 63–491 of NM_016665; Npas2, nt 1897–2344 of U77096; Bmal1, nt 360–619 of AB015203; Rev-erba, nt 1025–1326 of NM_145434; Rev-erbβ, nt 1094–1367 of U61124; Rorγ, nt 421–850 of U43508. Antisense riboprobes were prepared by in vitro transcription of linearized templates (Riboprobe Technology, Promega). RPA was carried out on 6–10 μg RNA using the RPAIII kit (Ambion), according to the manufacturer’s instructions, and run on denaturing acrylamide gel. Signals were quantitated by densitometry and normalized to β-actin, and the maximal value was set as 1.0.

Abs and flow cytometry

The following Abs were used for surface stainings: TCRO8 FITC (H57-597), CD8 PerCP, or FITC (53-6.7) (BD Biosciences, Mississauga, ON, Canada); CD4 allophycocyanin RM4-5 (BioLegend, San Diego, CA); CD44 allophycocyanin (IM7) and CD62L FITC (MEL-14) (Cedarlane, Burlington, ON, Canada); and Foxp3 PE (FJK-16s) and CD25 FITC (eBiosciences, San Diego, CA). Labeled cell samples were analyzed using a BD Biosciences FACSCalibur or FACSCanto. Raw data were analyzed with FlowJo 8.7.3 (Tree Star, Ashland, OR).

In vitro stimulation of LN T cells

On the second day in DD, inguinal, brachial, and axillary LNs were collected, mechanically dissociated, and stored on ice until manipulation. LN cells were stored on ice for a maximum of 24 h. To check that time spent on ice did not have an effect on our observations, experiments were performed with inverted experimental time points (e.g., CT6 after CT14 versus CT14 after CT6). LN cells (105/ml in PBS) were labeled with 0.5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C and washed three times in RPMI 1640 supplemented with 10% FBS. CFSE-labeled cells (2 × 106) were stimulated in 24-well plates coated with either 1 μg/ml (Figs. 2, 3, Supplemental Fig. 2) or 0.1 μg/ml (Fig. 4) anti-CD3 Ab (145-2C11; Cedarlane). In one series of experiments (Fig. 4), cells were costimulated with soluble anti-CD28 Ab (clone 37.51; Biolegend); a range of concentrations of 2–5 μg/ml anti-CD28 Ab was used in each experiment, and, in each case, we used data from the highest concentration that did not saturate the proliferative response. After 52–56 h, cells were harvested and stained with anti-CD4 and anti-CD8 Abs, followed by flow cytometry analysis. We chose to let cells proliferate for 52–56 h because this duration consistently allows sufficient proliferation without reaching saturation. Within each experiment, cells from each time point were stimulated at the same time of day and left to proliferate for the same amount of time. Relative levels of proliferation were calculated as a ratio of the percentage of cells having undergone three divisions or more to the percentage of undivided cells. These ratios were then expressed as the percentage of mean across time points (Fig. 2) and percentage of mean across time points and conditions (Figs. 3, 4, Supplemental Fig. 2).

Protein extraction and Western blot analysis

On the second day in DD, superficial inguinal, brachial, and axillary LNs were collected every 6 h over 24 h, and 105 cells were washed in PBS, pelleted, frozen on dry ice, and stored at −80°C. Total proteins were extracted in lysis buffer (10 mM HEPES [pH 8], 0.1 mM EDTA [pH 8], 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 4 mM spermidine, 0.7 mM DTT, 100 μg/ml BSA, 17% v/v glycerol, 0.5 mM PMSF, 0.1% Nonidet P-40, and Complete Mini protease inhibitor [Roche]). Extracts were run on SDS-PAGE and immunoblotted with anti–β-actin (Sigma-Aldrich), anti-protein kinase C (PKC) A-9 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-lymphocyte-specific protein tyrosine kinase (LCK), clone 3A5 (Santa Cruz Biotechnology), or anti-ZAP70 clone IE7.2 (Upstate, Lake Placid, NY), followed by anti-mouse HRP-conjugated Ab (Sigma-Aldrich), and finally ECL detection reagent (Perkin Elmer, Waltham, MA). ZAP70 levels were quantitated by densitometry, normalized to actin, and calculated as percentage of mean across 24 h, and the data from the four independent experiments were then averaged.

DC–OVA immunization

Bone marrow cells (2 × 106 cells/ml) were plated on 6-well plates in complete RPMI 1640, and GM-CSF (500 U/ml; Invitrogen) and IL-4 (250 U/ml; made in house) were added on days 0, 2, 3, and 6 of culture. To induce maturation of DCs, 1 μg/ml LPS was added on day 6. Also on day 6, 600 U/ml IL-2 (2BU; houses) was added overnight with 2 μg/ml OVA-257–264 peptide (Midwest Biotech, Fishers, IN). Cultures were underlayered on 14.7% Histodenz (Sigma-Aldrich) and centrifuged at 1200 × g for 20 min. DC–OVA were collected from the interface and washed three times with PBS before injection. Three mice per time point were injected i.v. with either 1.25 × 106 DC–OVA or sterile PBS. Injections at ZT18 were performed in dim red light. Cytotoxic T cell response to i.v. immunization is initiated in the spleen. Accordingly, following this type of immunization, we cannot detect any T cell expansion in the LNs (data not shown). Thus, after 7 d, the mice were sacrificed at the times indicated, and spleens were harvested and stored on ice until time of staining. Total spleen cell number and percentage of CD8+ T cells in spleen did not vary between the day and night time points (data not shown). Splenocytes were stained with K5–OVA tetramers (made in-house) and anti-CD3, anti-CD8, and anti-CD44 Abs, followed by flow cytometry analysis.

For restimulation and assessment of IFN-γ production, splenocytes from each mouse were cultured with 1 μg/ml OVA peptide in complete RPMI 1640 for 6 h at 37°C. For the last 2 h, 10 μg brefeldin A (Sigma-Aldrich) per ml of cells was added. Cells were fixed and then stained with anti–
IFN-γ Ab (clone XMG1.2; Invitrogen) or isotype control diluted in 0.5% saponin (Sigma-Aldrich). Cells were washed, cell surface stained with anti-CD8 and anti-CD4 Abs, and analyzed by flow cytometry.

Statistical analyses

All 24 h time courses (Figs. 1A, 2A, 5A, 5C, 5D, Supplemental Fig. 1C) were analyzed by one-way ANOVA (effect of time) with Bonferroni post hoc test where applicable. Proliferation assays at two time points with two conditions (Figs. 3B, 4, Supplemental Fig. 2) and clock gene expression in Clock mutant and wild-type (WT) mice were analyzed by two-way ANOVA (for effect of time and group) with Bonferroni post hoc tests. Data of DC-OVA immunizations (Fig. 6) were analyzed by two-tailed paired t test. Differences were considered to be significant if p < 0.05. All data of 24 h time courses were also analyzed to evaluate the circadian rhythmicity using a cosinor fit. In all cases, a harmonic regression model both a 24-h period and a 12-h period, as follows:

\[ Y_i = \mu + A \cos(2\pi t_i/24 - \phi) + A/2 - 2\pi \phi/24, \]

where \( Y_i \) is the \( i \)th smoothed value of proliferation for a given T cell subset at time \( t_i \), and \( \mu \) is the mean proliferation per subset. Circadian amplitude (\( A \)) is defined as the difference between the 24-h mean and the maximum along the cosinor curve, and \( \phi \) is the acrophase in clock hours.

For all other time courses, the fitting model used a 24-h period only, as follows:

\[ Y_i = \mu + A \cos(2\pi t_i/24 - 2\pi \phi/24), \]

where \( Y_i \) is the \( i \)th smoothed value of RNA or protein levels at time \( t_i \), and \( \mu \) is the mean levels across the time course. Circadian amplitude (\( A \)) is defined as the difference between the 24-h mean and the maximum along the cosinor curve, and \( \phi \) is the acrophase in clock hours. Residual error is assumed to be independent and to have a normal distribution \( e_i \sim N(0, \sigma^2) \). Each parameter was tested for significance with \( p < 0.05 \). The T statistic was used to evaluate whether the \( Y \) intercept, the amplitude, and the phase of the 24-h rhythm differed from zero.

Results

Clock gene expression and lymphocyte subsets in LNs over the circadian cycle

As a first step in studying circadian processes in the LNs, we evaluated clock gene expression. Mice were entrained on a LD cycle and then put in DD. Superficial LNs were collected every 6 h over 24 h on the second day in DD, and RNA was extracted. Quantitative PCR revealed that the clock genes \( Rev-erb \) and \( Per \) are rhythmically expressed in LNs (Fig. 1A), and that the phase of the rhythms is similar to that in other peripheral tissues (26, 34). In addition to \( Rev-erb \) and \( Per \), RPs also showed expression of the clock genes \( Per \) and \( Cry \), Rev-erb, \( Bmal \), \( Ror \), \( Npas2 \), and \( Dec \), in a circadian pattern very similar for both inguinal and brachial LNs (Fig. 1B). This reveals that the molecular clockwork is present in the LNs, suggesting circadian regulation of events in these organs.

Because previous research has shown rhythms of leukocyte populations in the blood of rodents and humans, we surveyed the relative amounts of different T cell subsets and B cells in LNs sampled from WT mice under DD. There were no variations across the day in the abundance of B cells, CD4+ or CD8+, naive, effector, activated, or regulatory T cells (Table I, Supplemental Fig. 1).

T cell proliferation presents a circadian rhythm

Given that the LN is the main site of T cell stimulation, we next investigated T cell proliferation potential over the 24-h cycle. LNs were collected from mice every 4 h over 24 h, under DD conditions. Cells were stained with the fluorescent cytosol marker CFSE and stimulated in vitro using an Ab directed against a TCR-associated polypeptide, CD3ε. This gives direct assessment of the ability of T cells to proliferate in response to Ag because anti-CD3 stimulation mimics Ag recognition by T cells. CD4+ and CD8+ T cells from LNs sampled from animals in the late day or in the night, and stimulated with anti-CD3, divided much more quickly than when LNs were taken in the early or mid day (Fig. 2A–E). As expected, CD8+ T cells proliferated more than CD4+ T cells. This rhythm is not due to the order of sampling, as it was also observed when day sampling was done after night sampling (Supplemental Fig. 2). This rhythm cannot be explained by a variation of T cells in the samples, because, as described above, the abundance of each T cell subset does not vary over the day in LNs.

In parallel to anti-CD3 stimulation, we also stimulated cells with PMA and ionomycin. There was no rhythm in the proliferation of CD8+ T cells stimulated by PMA/ionomycin (Fig. 2G, 2H), but interestingly, there was a low-amplitude rhythm in CD4+ T cells after PMA/ionomycin stimulation with a different profile than the one observed with anti-CD3 stimulation (Fig. 2F, 2H). An absence of rhythms in CD8+ T cells in response to PMA/ionomycin and a weak rhythm with a profile different from anti-CD3 stimulation in CD4+ T cells together suggest that the proliferation rhythm after triggering of the TCR is primarily due to mechanisms upstream of cell cycle regulation. This rhythmic control could be at the level of signaling events proximal to the intracellular tail of the TCR complex.

The T cell proliferation rhythm requires a circadian clock

The experiments described above were done with mice housed in DD, showing the endogenous nature of the rhythm. To confirm that the rhythmic T cell response is dependent on the circadian clock, we triggered proliferation of cells from mice mutant for the gene \( Clock \) (33). We first confirmed that rhythms of clock gene expression in the LNs of \( Clock \) mutant mice are blunted (Fig. 3A). Whereas LN cells sampled from WT mice at CT14 (early subjective night) are more responsive to mitogenic stimulation than cells sampled at CT6 (mid subjective day), cells from \( Clock \) homozygous mutant littersmates did not show such a day–night difference (Fig. 3B). This is not due to a difference in the relative amounts of cell subsets between WT and \( Clock \) mutant mice at CT6 or CT14 (Fig. 3C).

T cell proliferation remains rhythmic when the level of CD28 costimulation is enhanced

Both T cells and APCs are involved in T cell stimulation. Although we use a TCR-specific Ab in the above in vitro experiments, T cells receive the required costimulatory signals from APCs present in the culture. To shed light on the contribution of T cells in rhythmic proliferation, we enhanced the costimulatory signal using soluble anti-CD28. LN cells sampled from mice at CT14 are more responsive to mitogenic stimulation than cells sampled at CT6 regardless of whether the costimulatory signal comes only from the APCs included in the culture or with the enhancement of costimulation using soluble anti-CD28 Ab (Fig. 4). These results indicate that the effect of costimulation has less of an influence on rhythmic proliferation than do factors inherent to the T cells themselves.

Expression of TCR and downstream signaling molecules over the 24-h cycle

We then examined T cell proliferation-relevant molecules that could be under circadian control. Our in vitro T cell proliferation experiments indicated that the rhythm of T cell proliferative response may be based on changes in the TCR-proximal signaling (Fig. 2). Thus, we first investigated cell surface expression of the TCR itself, and showed that it does not present a circadian rhythm in LNs (Fig. 5A). Thus, the rhythm in proliferation is not due to a variation in the number of TCRs over the day.
We next examined key molecules downstream of the TCR. After the engagement of the TCR, LCK is activated and phosphorylates the ζ-chains of the TCR-associated CD3 molecule. The cytosolic tyrosine kinase ZAP70 is then recruited and phosphorylated, and plays a pivotal role in initiating downstream signaling, including PKCε activation (9). LNs were collected every 6 h over 24 h under DD conditions, and protein extracts were immunoblotted for LCK, PKCε, and ZAP70. Although LCK and PKCε did not present a circadian rhythm (Fig. 5B), ZAP70 protein expression was found to be rhythmic with peak levels at CT8 (Fig. 5B, 5C). Notably,

![Figure 1](URL)  
**FIGURE 1.** Clock genes are rhythmically expressed in LNs and show similar expression in inguinal and brachial LNs. A, LNs were taken from mice in DD at the indicated CT. *Rev-Erbα* and *Per2* RNA levels were analyzed in samples from individual mice by quantitative PCR, relative to β-actin. *n* = 4 mice per CT. Analysis by one-way ANOVA revealed a significant effect of time for *Rev-Erbα* (*p* < 0.01; Bonferroni post hoc tests for CT2 versus CT8 and CT8 versus CT14, *p* < 0.05; CT8 versus CT20, *p* < 0.001) and for *Per2* (*p* < 0.01; Bonferroni post hoc tests for CT14 versus all other CTs, *p* < 0.05). Cosinor analysis (gray lines) revealed a significant 24-h rhythm for both *Rev-Erbα* (*p* < 0.0001) and *Per2* (*p* < 0.0001). The experiment was done twice, with similar results. B, Inguinal (■) and brachial (△) LNs were collected, as in A, and used to prepare RNA. Equal amounts of RNA from six mice were pooled for each time point and analyzed by RPA for the indicated clock genes. RNA curves were quantified by densitometry and normalized to β-actin.

We next examined key molecules downstream of the TCR. After the engagement of the TCR, LCK is activated and phosphorylates the ζ-chains of the TCR-associated CD3 molecule. The cytosolic tyrosine kinase ZAP70 is then recruited and phosphorylated, and plays a pivotal role in initiating downstream signaling, including PKCε activation (9). LNs were collected every 6 h over 24 h under DD conditions, and protein extracts were immunoblotted for LCK, PKCε, and ZAP70. Although LCK and PKCε did not present a circadian rhythm (Fig. 5B), ZAP70 protein expression was found to be rhythmic with peak levels at CT8 (Fig. 5B, 5C). Notably,

### Table I. Lymphocyte subsets do not vary over the day in mouse LNs

<table>
<thead>
<tr>
<th>Subset</th>
<th>CT2 (×10⁶ Cells)</th>
<th>CT8 (×10⁶ Cells)</th>
<th>CT14 (×10⁶ Cells)</th>
<th>CT20 (×10⁶ Cells)</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4⁺ T cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10.5 ± 0.3</td>
<td>11.7 ± 0.3</td>
<td>11.4 ± 0.3</td>
<td>10.8 ± 0.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Effector</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Naive</td>
<td>7.4 ± 0.1</td>
<td>8.7 ± 0.1</td>
<td>8.5 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Regulatory</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>CD8⁺ T cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.3 ± 0.1</td>
<td>9.7 ± 0.1</td>
<td>9.9 ± 0.1</td>
<td>8.7 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Effector</td>
<td>0.5 ± 0.02</td>
<td>0.3 ± 0.01</td>
<td>0.4 ± 0.02</td>
<td>0.4 ± 0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Naive</td>
<td>5.9 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>B cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11.9 ± 0.1</td>
<td>11.8 ± 0.2</td>
<td>11.9 ± 0.2</td>
<td>12.1 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Superficial LNs were taken from mice in DD at CTs. Cells of individual mice were stained for CD4, CD8, CD62L, and CD44; or CD4 and CD25; or IgM and B220. Naive T cells are those that stain CD4⁺ or CD8⁺ and CD44lowCD62Lhigh; effector T cells are CD4⁺ or CD8⁺ and CD44highCD62Llow; regulatory T cells are CD4⁺CD25⁺; and mature B cells are IgM⁺B220⁺. The experiment was done three times, except for staining for CD62L and CD44 (twice), with three to four mice each time. All numbers shown are mean ± SEM for the two to three experiments grouped, i.e., for 7–11 mice per time point (individual data points are shown in Supplemental Fig. 1C). The values in the table are the absolute number of cells in three LNs. All variations across CTs are nonsignificant by one-way ANOVA (rightmost column).
Zap70 mRNA is also rhythmically expressed (Fig. 5D), with a profile similar to that of the protein, which indicates that this molecule might be controlled at the transcriptional level by a T cell circadian clock. A ZAP70 rhythm might underlie rhythmic T cell function. However, the high levels of proliferation potential of LN T cells persist throughout the late day and night, and present a bimodal profile (Fig. 2D,2E), implying that additional molecular mechanisms are probably involved.

In vivo Ag-specific T cell response varies according to the time of immunization

We next investigated T cell activation in response to Ag presentation in vivo. Bone marrow-derived DCs loaded with the class I-restricted OVA peptide (DC-OVA) were injected in mice either in the middle of the day (ZT6) or in the middle of the night (ZT18). Seven days later, mice were sacrificed at the same time point as injection. OVA-specific CD8+ T cells were quantified in the spleen using the Kb-OVA tetramer. Mice injected at ZT6 showed a higher proportion of OVA-specific CD8+ T cells than mice injected at ZT18 (Fig. 6A,6B). To distinguish whether group differences were due to the injection or dissection time, we also injected mice at ZT6 and sacrificed them 7 d later, but at ZT18, and vice versa. Regardless of dissection time, mice injected at ZT6 showed a markedly higher response to immunization than mice injected at ZT18 (Fig. 6C). To see whether the T cells activated by DC-OVA acquired effector functions, splenocytes were restimulated with

**FIGURE 2.** LN T cell proliferative response to anti-CD3 or PMA/ionomycin stimulation. LNs were taken from mice in DD at six CTs. Cells from four mice per time point were pooled, labeled with CFSE, and plated on anti-CD3–coated wells (1 μg/ml). Cells were collected after 52–56 h in culture, and stained with anti-CD4 and anti-CD8 Abs, followed by flow cytometry analysis. A, CFSE fluorescence intensity profiles gated on CD4+ or CD8+ T cells. The experiment was done three times with similar results, and a representative experiment is shown. B and C, Averaged data of three independent experiments (including the one shown in A) for CD4+ (B) and CD8+ (C) T cells. A proliferation index was calculated as the percentage of cells that have divided three times or more, divided by the percentage of undivided cells. Data are expressed as a percentage of the mean of this index over six CTs (left panels, one-way ANOVA; CD4+, p < 0.05; CD8+, p > 0.05) or after grouping in two time bins (right panels, t test; *p < 0.05). D and E, Dual harmonic regression fit of the percentage of the mean of the data represented in B and C (CD4+; cosinor 24 h, p < 0.002; cosinor 12 h, p < 0.02; CD8+; cosinor 24 h, p < 0.01; cosinor 12 h, p < 0.05). F and G, Data for the proliferation of T cells (compilation of the same three experiments as in B) in response to PMA/ionomycin; CD4+ (F), CD8+ (G). Data were analyzed by one-way ANOVA; CD4+, p = 0.03; CD8+, p > 0.05. H and I, Dual harmonic regression fit of the percentage of the mean of the data represented in F and G (CD4+; cosinor 24 h, p < 0.01; cosinor 12 h, p > 0.05; CD8+; cosinor 24 h, p > 0.05; cosinor 12 h, p > 0.05).
the OVA peptide for 6 h, followed by intracellular staining to detect IFN-γ. There were more IFN-γ–producing cells when animals were initially injected in the day than in the night (Fig. 6D, 6E), paralleling the results obtained with tetramer staining. The fold difference is proportional for these two markers of T cell expansion and activation. Thus, we can also conclude that the T cell activation day–night difference is not due to a more efficient staining of splenic T cells with tetramer at ZT6. These data indicate that T cells respond differentially to Ag presentation depending on time of day in vivo.

FIGURE 3. Levels of clock gene RNAs, T cell proliferation, and T cell subsets in LNs of Clock mutant mice. A, LNs were taken from four WT (+/+, ■) and four Clock mutant mice (c/c, □) in DD at the indicated CTs. RNA was extracted for each mouse and pooled using equal amounts from each mouse within each time point, and quantitative PCR was performed for Rev-Erbα and Per2, relative to β-actin. In WT LN RNA, rhythmic expression of Rev-Erbα and Per2 was significant in a 24-h cosinor analysis (p < 0.002 and p < 0.001, respectively). Cosinor analyses (gray lines) for Clock mutant mice LN RNA were nonsignificant for both Rev-Erbα and Per2. B, LNs were taken from WT (+/+) and Clock mutant (c/c) mice in DD at the indicated CTs. Cells from four mice per time point were labeled with CFSE and plated in anti-CD3 Ab-coated wells (1 μg/ml). Cells were collected after 54 h in culture and stained for CD4 and CD8, followed by flow cytometry analysis. The experiment was done three times, with similar results; data were expressed as a percentage of the mean, and a composite of the three experiments is shown (mean ± SEM). Data were analyzed by two-way ANOVA with Bonferroni post hoc tests; day–night difference for WT/CD4+*, p < 0.01; and WT/CD8+, p < 0.002. Clock mutant mice, both cell types, p > 0.05; comparison between genotypes, p > 0.05. C. For each CT and genotype, CD4/CD8 profile (left), CD44/CD62L profile gated on CD4+ or CD8+ T cells (middle), and CD25 profile gated on CD4+ T cells (right) are shown. The percentage of naive T cells (CD44lowCD62Lhigh), activated/memory T cells (CD44highCD62Llow), and CD25+ regulatory T cells is shown. The experiment was done three times, with similar results, and a representative experiment is shown.
Discussion

The results presented in this study reveal clear variations over the day of T cell function both in vitro and in vivo. When stimulated with a mitogenic signal in culture, T cells exhibit a circadian rhythm of proliferation levels. Notably, this is not due to variation of lymphocyte subsets in the LN or of CD28 costimulation from APCs, but might depend on the circadian control of TCR-dependent signaling proteins such as ZAP70, whose levels present a circadian rhythm. As shown with DC–ova immunizations, T cell response to Ag presentation also presents a time dependency in vivo.

Previous work has uncovered a rhythm of proliferation of blood T cells in humans, with maximal levels in the night (35). Proliferation of rat LN cells in response to Con A, a lectin that activates T cells by bridging their TCRs, was also shown to present a daily variation, but with variable phases and amplitudes across conditions and studies (36–38). However, in the latter case, rats were first treated with Freund’s adjuvant, which activates the innate response, and the amplitude of the proliferation rhythm was greatly reduced without this prior stimulation. The same authors found rhythms of lymphocyte subsets in rat submaxillary LNs (36, 37) (but not in other studies; see Ref. 39 for example), which contrasts our data showing constant numbers of T cell subsets and B cells in mouse superficial LNs. Melatonin can alter the balance of T cell subsets (40). Because rats produce a high-amplitude melatonin rhythm (41), whereas C57BL/6 mice produce very low

FIGURE 4. Enhancing the level of costimulation in vitro with anti-CD28 has no effect on the day–night difference in T cell proliferation. LNs were taken from mice in DD at the indicated CTs. Cells from four mice per time point were pooled, labeled with CFSE, and plated in anti-CD3 Ab-coated wells (0.1 μg/ml). Cells were costimulated with soluble anti-CD28 Ab. A range of conditions from 2 to 5 μg/ml was used in every experiment, and in each case we used data from the highest concentration that did not saturate the proliferative response. Cells were collected after 56 h in culture and stained for CD4 (left panel) and CD8 (right panel), followed by flow cytometry analysis. The experiment was done three times, with similar results; data were expressed as a percentage of the mean, and a composite of the three experiments is shown (mean ± SEM). Data were analyzed by two-way ANOVA: day–night difference for CD4+ CT6 versus CT14 comparison, *p < 0.02; group difference, p > 0.05; group–time interaction, p > 0.05; day–night difference for CD8+ CT6 versus CT14 comparison, *p < 0.03; group difference, **p < 0.01; group–time interaction, ***p < 0.01.

FIGURE 5. Expression of TCR and downstream signaling molecules over the day in LNs. LNs were taken from WT mice in DD at the indicated CTs, and then processed for surface staining, Western blotting, or quantitative PCR. A, Cells from four individual mice per time point were stained using anti-TCRαβ Ab, followed by flow cytometry. The mean fluorescence intensity for lymphocytes is plotted with each point representing a single mouse. Analysis by one-way ANOVA showed no significant daily variation of the expression level of TCRαβ (p > 0.05). B, Protein extracts pooled from four mice were resolved by SDS-PAGE and immunoblotted with Abs for LCK, PKC, ZAP70, and ACTIN. The experiment was done four times, with similar results, and a representative blot is shown. C, Quantification of ZAP70 protein expression from four experiments (the one shown in B and three other experiments), with similar results; data were expressed as a percentage of the mean, and a composite of the four experiments is shown. Analysis by one-way ANOVA revealed a significant effect of time (p < 0.05; Bonferroni post hoc test for CT8 versus CT20, p < 0.05). Cosinor analysis (gray line) revealed a significant 24-h rhythm (p < 0.0001). D, Quantitative PCR was done for Zap70, relative to β-actin, on RNA extracted from LNs of four individual mice per CT. One-way ANOVA showed no significant effect of time (p = 0.17). Cosinor analysis (gray line) revealed a significant 24-h rhythm (p < 0.0001). The experiment was done twice, with similar results (but with significant effect of time in ANOVA for the other experiment).
levels of this hormone (42), an effect of melatonin may explain the difference observed between the studies.

Notably, these previous studies were all conducted under alteration of light and dark, that is, in conditions that make it impossible to distinguish between endogenous control (e.g., by the clock) and environment-driven daily variations. Our in vitro T cell proliferation experiments were conducted with mice housed in DD, thus eliminating the possibility that the phenomenon results from light-driven processes. Consistent with a control by the circadian system, LN T cells from Clock mutant mice did not display the day–night difference in T cell proliferation. These mutant mice have a mutation in the gene encoding the protein CLOCK, leading to the skipping of one exon and to a dominant-negative form of this transcription factor (43, 44). These mice display a long free-running period in DD, and eventually arrhythmicity after 2–3 wk (33). Moreover, in these mutants, the expression of the genes controlled by the CLOCK/BMAL1 dimer is blunted (45–47), and the amplitude of the corticosterone rhythm is reduced (48) or abolished (46) depending on the genetic background. These mutant mice also display shorter total sleep duration and reduced recovery sleep after sleep deprivation (49). Both corticosterone (21, 50–52) and sleep (10, 53) are known to have an impact on the immune response. Yet, a detailed analysis of immune responses of Clock mutant mice has not been reported. The amplitude of leukocyte count rhythms is reduced in the peripheral blood of Clock mutant mice (48, 54). Notably, changes in cell numbers are unlikely to explain the proliferation described in this work, as we found no difference in levels of lymphocyte subsets in LNs of Clock mutant versus WT mice.

Because Clock mutant mice lack a robust functional clock in all cell types, our experiment does not indicate which cell types or which, if any, humoral or neuronal cues contribute to circadian control of T cell response. Our results show a clear circadian variation in T cell proliferation in whole LN extracts, containing both T cells and APCs. Hayashi et al. (32) and Keller et al. (30) showed rhythmic clock gene expression in macrophages, which are one type of APCs, and rhythms in phagocytic activity and cytokine release function, respectively. Thus, the contribution of either a T cell clock, an APC clock, or other clocks in the organism (e.g., the SCN) to T cell proliferation remains to be determined. To address the potential contribution of APCs to the in vitro T cell proliferation rhythm, we performed an experiment in which the CD28 costimulatory signal was strongly enhanced. CD28 engagement is a key signal for the priming of naive T cells. In this experiment, high CD28 engagement mimicked a strong and constant CD28 costimulation signal from APCs. Even under these conditions, T cells still exhibited a marked day–night difference in their capacity to proliferate. These data reduce the likelihood of a rhythmic contribution from APCs, and indicate that the T cells themselves have the intrinsic ability to respond differentially to TCR trigger according to the time of day.

The results we show illustrating a strong day–night difference in response to immunization in vivo provide additional insights. The DCs injected both at ZT6 and ZT18 were from the same culture of
matured bone marrow cells collected at a single time point from donor mice. Given that the DC–OVA immunization approach bypasses events prior to Ag presentation to T cells, the effect we see in vivo is most likely not due to an APC-intrinsic clock. For the same reasons, this day–night difference of response to DC–OVA immunization is probably not explained by the stronger innate responses observed in the rest period (11, 12, 30, 53). Rather, the day–night difference probably relies either on a T cell-intrinsic factor, on the effect of a systemic cue acting on T cells (immunomodulatory hormones, autonomic signals, sleep/wake cycles, spleen environment), or on a combination of rhythmic T cell and rhythmic systemic cue processes.

Such a multilevel additive circadian regulation has been described for other physiological processes and tissues. For example, the high-amplitude rhythm of corticosterone synthesis and release depends on both SCN central clock function and the local adrenal gland clock (55, 56). Also, circadian regulation in the liver relies on both the hepatocyte clock and systemic cues (57, 58). A complex, organism-wide phenomenon such as the immune response is well placed for a multigated circadian control. Within this complex response, a central event like the T cell response to Ag and proliferation is a prime position for such a point of control.

As an initial step in identifying points of circadian control of T cell function, we assessed the abundance over the day of several proteins downstream of the TCR. We found that Zap70 protein levels follow a circadian rhythm. This is paralleled by a rhythm in the mRNA, suggesting regulation at the transcriptional level by a T cell clock machinery. Interestingly, the search for putative transcription factor binding sites in the Zap70 gene revealed two regions of high similarity between mouse and human genomes that contained E-boxes, DNA elements that can be bound by the clock transcription factors CLOCK and BMAL1 (1), just upstream of the first exon and within the first intron (data not shown). Could a rhythm of Zap70 protein levels lead to a daily variation of the capacity of T cells to proliferate? Variations in abundance of proteins modulating TCR responses were shown to impact the amount of engagement of the TCR in complexes with MHC-Ag or the strength of signaling downstream of the TCR (59). Changes in expression levels of Zap70 itself were shown to be functionally relevant in vivo: in the thymus, Zap70 protein levels vary throughout thymocyte development, and these changes in abundance underlie the precise timing of development of single-positive CD4+ and CD8+ T cells (60). Thus, the circadian rhythm of Zap70 levels in LN T cells could contribute to the rhythm of proliferation potential. Nevertheless, the high levels of proliferation potential persist throughout the late day and night, implying that other mechanisms are likely to be involved. Additional levels of regulation could include the following: rhythms of other proteins downstream of the TCR, rhythms of T cell responsiveness to cytokines or to APC costimulatory signals, rhythms in the levels to which signaling pathways can be activated, or a daily variation in the spatiotemporal regulation of the TCR or TCR-associated factors.

A time-of-day preference for immune potentiation could be advantageous to optimize the response at phases of the day during which a pathogen is most likely to be encountered. Alternatively, a period of low responsiveness could prevent a hypersensitivity that could lead to deleterious complications, including allergy and autoimmunity. Beyond this, circadian control of T cell proliferation and the adaptive immune response provides a new paradigm for exploration of disease susceptibility. In particular, a higher incidence of cancer was shown in conditions in which circadian rhythms were disrupted (61–63), and in particular in shift workers (64–66).

Furthermore, our striking observation that the time of immunization has a strong impact on the magnitude of the adaptive immune response as measured by looking at T cell activation, proliferation, and acquisition of effector functions many days later, lends serious support to the novel idea that the efficiency of vaccination might benefit from careful selection of the time of administration. A few pilot studies with subjects receiving vaccine for hepatitis A or B or influenza have indicated that, indeed, the outcome of vaccination could depend on the time of administration (67–69). Our work gives a clear and controlled demonstration of this concept and provides an easily tractable system to dissect the mechanisms at play.

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Disclosures

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