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Photoperiod alters macrophage responsiveness, but not expression of Toll-like receptors in Siberian hamsters

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Abstract

Defense against pathogens is a critical component of comparative and ecological biology. However, pathogen recognition, a process necessary for the facilitation of systemic immune response, remains understudied in a comparative context, yet could provide insight into how the immune system interacts with pathogens in variable environments. We examined pathogen recognition by macrophages in relation to an ecological variable, day length, in Siberian hamsters (*Phodopus sungorus*). Because peritoneal macrophages collected in long, summer-like day lengths are more responsive to a lipopolysaccharide (LPS) challenge compared to macrophages collected during short, winter-like day lengths, we hypothesized that these functional differences are mediated by variation in pathogen recognition, which occurs through binding to Toll-like receptors (TLRs). We predicted that expression of TLR2 and 4, the receptors that bind and respond specifically to LPS, would be upregulated in long vs. short days, and that expression of these receptors would reflect macrophage responsiveness to LPS. Macrophages collected during long days were again more responsive to LPS challenge compared to short-day macrophages; however, TLR2 and TLR4 expression was similar between photoperiods and were unrelated to our measure of macrophage responsiveness suggesting that other downstream intracellular mechanisms may be responsible for photoperiod-based variation in macrophage responsiveness in this species.

Keywords: Photoperiod; Immunity; Seasonal; Toll-like receptor; Lipopolysaccharide; Ecoimmunology

1. Introduction

Organisms have developed complex systems for recognizing and eliminating pathogens, consisting of cascades of cellular interactions that involve multiple cell types (Abbas et al., 2000). Vertebrate immunity has become a major focus of comparative and ecological literature (Martin et al., 2006) because an understanding of the mechanisms and costs associated with immunological defense can lend insight into the evolution of other complex life history traits (Sheldon and Verhulst 1996). Until recently, however, ecological immunology studies have been limited to non-specific and sometimes rough measures of immune function. The development of molecular approaches to immunological questions in non-conventional study species has only just begun to provide a more in-depth understanding of the immune system as well as its potential for ecological implications.

A majority of ecological studies focus on the efficacy of the two subdivisions of the vertebrate immune system — the innate and adaptive arms (reviewed in Martin et al., 2006). Very few studies, however, address questions of microbe recognition, a process responsible and necessary for the stimulation of either immunological arm. Following pathogen invasion, the pathogen must initially be recognized by the first line of immunological cells that includes macrophages, neutrophils, and natural killer cells. Macrophages are particularly important to overall immunity because they are not only initially responsible for phagocytosis and cytotoxic attack on pathogens, but also alert other cells of the immune system and prepare them for the assault (Gordon, 2002). Macrophages are capable of reacting to a variety of pathogen types using pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), molecules that are broadly shared by pathogens but are distinguishable from host molecules (Aderem and

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Ulevitch, 2000). Toll-like receptors (TLRs) constitute a family of 13 type I transmembrane proteins that function as PRRs, and effectively recognize a plethora of pathogens, including but not limited to Gram negative and positive bacteria, flagellins, single-stranded RNA, fungi, and viral glycoproteins (reviewed in Aderem and Ulevitch, 2000; Lien et al., 2000). Following TLR-mediated pathogen recognition, macrophages release a suite of cytokines that direct the eventual systemic response to the pathogen, often resulting in inflammation in the periphery and brain as well as fever (Burke and Lewis, 2002; Gordon, 2002). Thus, recognition of pathogens by TLRs is important in the direction of immunological responses to those pathogens.

Toll-like receptors have been identified throughout the vertebrate subphylum, for example in fish (Stafford et al., 2003; Meijer et al., 2004), pigs (Muneta et al., 2003), birds (Farnell et al., 2003), and amphibians (Ishii et al., 2007). Additionally, these studies support a comparable role for Toll-like receptors in these species, as TLRs are shown to be genetically upregulated in response to a challenge and stimulate the immune system to respond (Farnell et al., 2003, Muneta et al., 2003; Stafford et al., 2003). Thus, it may be useful to examine TLR function in an ecological context do determine how variation in pathogen recognition relates to environmental conditions.

The Siberian hamster is an excellent ecologically-relevant species on which to focus immunological studies, because they are seasonally breeding rodents that experience numerous physiological changes in response to short, winter-like day lengths, including dramatic decreases in body mass, adipose tissue mass (Bartness et al., 2002) and testicular mass (Bilbo et al., 2002), as well as a suite of immunological parameters (Yellon et al., 1999, Drazen et al., 2001; Zhou et al., 2002). Studies in Siberian hamsters suggest that macrophage-mediated immunological events, in particular, vary seasonally. For example, hamsters maintained in long day lengths (LD) that mimic summer conditions have significantly higher levels of interleukin-6 (IL-6) in circulation and produce higher fevers compared to animals maintained in short-day lengths (SD) that mimic winter conditions (Bilbo et al., 2002). Both IL-6 production and febrile responses are mediated in part by macrophages (Abbas et al., 2000). In addition, LD hamsters exhibit increased macrophage-mediated phagocytosis activity (Yellon et al., 1999), and increased oxidative burst activity (Yellon et al., 1999) compared to SD hamsters.

A recent study suggests that these photoperiod differences may be mediated by Toll-like receptors. Activation of Toll-like receptors stimulates macrophages to produce the pro-inflammatory cytokine, tumor necrosis facror α (TNFα)(Aderem and Ulevitch, 2000). Thioglycollate-elicited peritoneal macrophages cultured from LD hamsters produced significantly more TNFα after a challenge with a bacterial component known to stimulate specific Toll-like receptors, lipopolysaccharide (LPS) compared to macrophages cultured from SD hamsters (Prendergast et al., 2003). Thus, we hypothesized that expression of the TLRs known to recognize and respond to LPS, TLR2, and TLR4 (Morissette et al., 1999; Lien et al., 2000), would vary according to day length in Siberian hamsters, and would relate directly to TNFα production by the same macrophages. By examining molecular mechanisms associated with pathogen recognition in an ecologically-relevant species, we hoped to heighten the understanding of how the vertebrate immune system interacts with the environment.

2. Materials and methods

2.1. Animals

Thirty-two adult male Siberian hamsters (4 to 6 months of age) from our breeding colony were used in this study. Animals were housed individually at a constant temperature of 21±2 °C and humidity of 50±5%, and ad libitum access to food and water. Hamsters were housed in one of two treatments: long day lengths (LD) consisted of 16 h light:8 h dark and short-day lengths (SD) consisted of 8 h light:16 h dark. All hamsters were maintained in their respective photoperiods for 11 weeks after which hamsters were killed and the following was collected: body mass, paired testes for testicular mass, spleen for splenic mass, epididymal adipose tissue (EAT) for an estimation of adipose tissue mass, and peritoneal macrophages for RNA extraction. Hamsters were killed and tissues and macrophages harvested between 1000 and 1100 h EST on two consecutive days, 4 h prior to the onset of the dark cycle for both groups. This time period was chosen because it represents the exact middle of the light phase for the short-day hamsters (hour 4 of a total 8 h of light), and is the minimum amount of time after the onset of sleep and before the onset of wakefulness necessary to avoid physiological changes associated with the sleep/wake cycle in both groups (Albers et al., 1985). Over the course of the experiment, 5 hamsters died of unknown causes, lowering the sample size to 13 LD and 14 SD animals. Additionally, when hamsters were killed, tissue weights were only collected from approximately half of the animals, because tissues from the other half were immediately utilized for other analyses. Thus, sample sizes for tissue masses were 7 in LDs and 8 in SDs.

2.2. Peritoneal macrophage extraction and LPS challenge

Hamsters were injected intraperitoneally with 3 ml of sterile 4% sodium thioglycollate to stimulate peritoneal macrophage activation and recruitment. Three days later, hamsters were killed by cervical dislocation, and thioglycollate-elicited macrophages were extracted according to techniques described in Prendergast et al. (2003). Briefly, after hamsters were killed, the abdominal skin was deflected and 8 ml of sterile Dulbecco's Modified Eagle Medium (DMEM) was infused into the peritoneal cavity using a 10 ml syringe. The peritoneal cavity was gently agitated to loosen adherent macrophages and DMEM containing elicited macrophages was withdrawn. Macrophages were washed several times with sterile DMEM and resuspended in supplemented DMEM (containing 5% fetal bovine serum and 1% penicillin/streptomycin) and counted using a hemacytometer. Macrophages (1×10^6 cells) were aliquoted into sterile 6-well culture plates, 3 ml sterile supplemented DMEM was added, and plates were incubated

in a 5% CO_2 incubator at 37 °C for 1 h to permit monolayer formation. Macrophage monolayers were washed twice with sterile DMEM and 5 ml fresh supplemented media was added. Macrophages were then treated with either LPS (1 μg/ml, 011B4, in 50 μl culture media, total concentration after addition was 0.2 μg/ml) or a control (25 μl culture media) treatment. Plates were incubated for two hours at 37 °C after which 400 μl of cell culture supernatant was removed and frozen immediately at −80 °C until assayed for TNFα. Previous studies examining the time course of TNFα production in this species has shown that peak production occurs at 2 h post-LPS exposure (Prendergast et al., 2003).

TNFα in cell culture was measured according methods described in Prendergast et al. (2003) using an ELISA (rat TNFα, Ultra-Sensitive, Biosource, Camarillo, CA). All samples were run in duplicate and a standard curve was included on the assay plate for extrapolation of experimental TNFα values. The optical density of all wells was determined at a wavelength of 450 nm using a Bio-Rad microplate reader. During the macrophage extraction process, the cavity of one hamster burst, preventing macrophage collection. Thus sample sizes for this portion of the experiment are 13 per treatment group. In addition, the number of control samples was limited by space on the ELISA plate. Thus only 11 control macrophage samples, a roughly even distribution between the treatment groups, were run.

2.3. Sequencing Siberian hamster TLR2 and TLR4

Both the spleen and one aliquot of washed peritoneal macrophages (1×10^6 cells) were stored in RNA later and frozen at −80 °C until RNA extraction. Total RNA was later extracted from macrophages using the High Pure RNA extraction kit from Roche Applied Science (Indianapolis, IN) and from homogenized spleen samples using the RNeasy miniprep kit from Qiagen (Darnestown, MD) according to the manufacturers' instructions. To sequence the Siberian hamster TLR2 and TLR4 genes, cDNA was created via reverse transcription of 2 μg hamster macrophage RNA using MMLV Reverse Transcriptase Enzyme from Invitrogen (Carlsbad, CA). The following primer sequences were designed for Siberian hamster TLR2 and TLR4 based on rat, mouse, and canine sequences that shared >90% homology using Primer Express Software (Applied Biosystems, Foster City, CA): TLR2: forward 5′GAGTGAGTGGTGGCAAGTATGAAC3′, reverse 3′GGGCCACTCCAGGTAGGTCT5′, TLR4: forward 5′GCTGCATTCCCTCAAGACTC3′, reverse 3′TTCTCACC-CAGTCCTCATCC5′. PCR was conducted on pooled hamster macrophage cDNA using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) and PCR products visualized on a 1% TAE-agarose gel containing ethidium bromide. PCR products were purified (Qiaquick kit, Qiagen), subcloned using a TOPO cloning kit (Invitrogen), and sequenced. The resulting sequences that were >95% homologous to the published *Mus* TLR4 and TLR2.

2.4. Measurement of TLR2 and TLR4 expression in elicited peritoneal macrophages

Primers and probes for TLR2 and TLR4 were designed for quantitative PCR using Primer Express. Probes labeled with 6-FAM and primers were ordered from Applied Biosystems (Foster City, CA) and Invitrogen respectively: TLR2 forward 5′

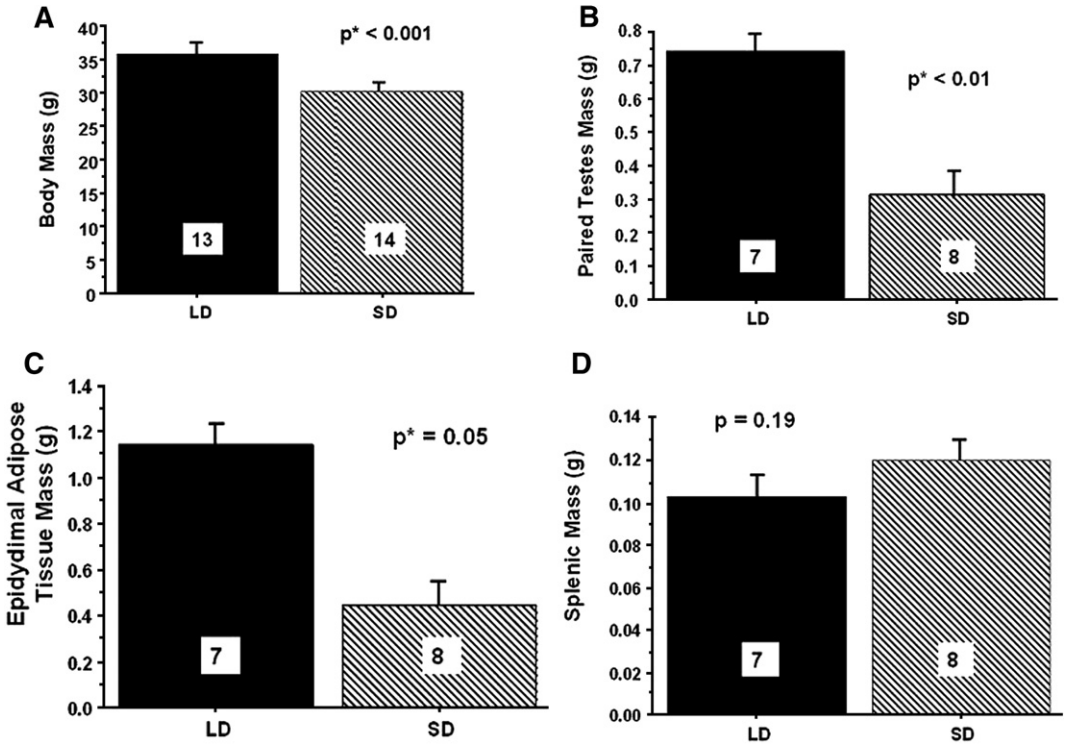

Fig. 1. Mean (±SE) (A) body mass, (B) paired testes mass, (C) epididymal adipose tissue mass, and (D) splenic mass of animals maintained in either LD (16 h light:8 h dark) or SD (8 h light:16 h dark) conditions for 11 weeks.

CTGGAGCCCATTGAGAAGAAAG, reverse 5′TCATTATCTTGCGAAGCTTGCA, probe 5′CATCCCCCAGCGCT, TLR4 forward 5′GGTCAAGGACCAGAGGTTGT, reverse 5′ CTTCATGTCTACAGGTGTTGCACA, probe 5'CTAGTGAACACTGAACAAAT. A Taqman 18S ribosomal RNA primer and probe set labeled with VIC (Applied Biosystems) was used as the control gene for relative quantification. Amplification was performed using an ABI Sequencing Detection System and Taqman Universal PCR Mastermix. 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 s and 60 °C for 1 min. Individual samples were run in duplicate with a relative standard curve consisting of serially diluted pooled *P. sungorus* macrophage cDNA (1:1, 1:10, 1:100, 1:1000). Relative gene expression was determined according to the standard curve following normalization to 18S gene expression. Due to difficulties with the extraction of clean RNA (assessed via spectrographic analysis with a Nanodrop spectrophotometer) from macrophages, only macrophages from which we obtained a good concentration of clean RNA were used for the amplification of the products, decreasing the sample sizes to the numbers shown in the figures.

2.5. Data analysis

All statistical analyses were performed by Statview software (Version 5.0.1, SAS Institute, Inc.). Group comparisons were performed using ANOVA or ANCOVA tests. Since body mass contributed significantly to measures of paired testes mass and epididymal adipose tissue mass, we used body mass as a covariate in those analyses. Comparisons of relative expression between genes of interest and between splenic tissue and macrophages were conducted using paired *t*-tests. The relationships between macrophage TLR2 and TLR4 expression and TNFα production after LPS challenge were determined using simple regressions. Post hoc power analyses were conducted using GPOWER (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/).

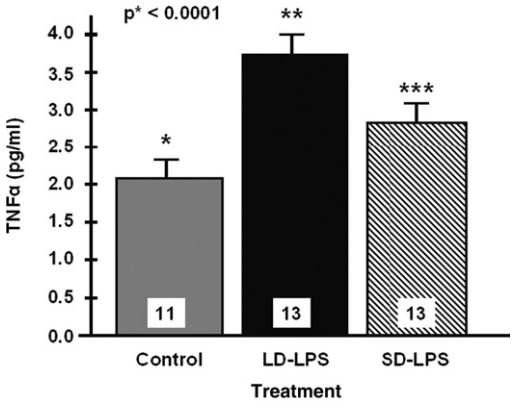

Fig. 2. Mean (±SE) tumor necrosis factor alpha concentrations in cell culture supernatant collected from macrophages that have been cultured with either LPS or control cell culture media. Cultured macrophages were collected from individuals maintained in either LD (16 h light:8 h dark) or SD (8 h light:16 h dark) conditions for 11 weeks. Statistical significance ($p \leq 0.05$) is indicated by different numbers of asterisks.

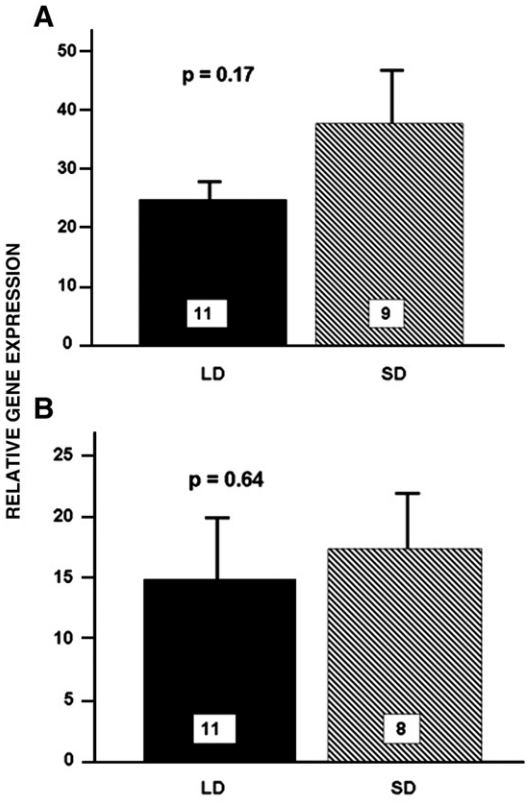

Fig. 3. Mean (±SE) expression of peritoneal macrophage (A) TLR2 and (B) TLR4 relative to expression of 18S ribosomal RNA in animals maintained in either LD (16 h light:8 h dark) or SD (8 h light:16 h dark) conditions for 11 weeks.

3. Results

3.1. Body mass and organ weights

In accordance with previous experiments, hamsters housed in LD and SD conditions differed phenotypically (Fig. 1). LD hamsters were significantly heavier ($F_{1,27}=14.74$, $p<0.001$) (Fig. 1A), had significantly more epididymal adipose tissue ($F_{2,15}=5.087$, $p=0.05$)(Fig. 1B), and had significantly larger paired testes masses ($F_{2,15}=12.96$, $p<0.01$)(Fig. 1C) than SD hamsters. Spleen mass showed a tendency to be larger in SD hamsters, but this difference was not significant ($F_{1,15}=1.923$, $p=0.19$)(Fig. 1D).

3.2. TNFα production by elicited peritoneal macrophages

Macrophages cultured from LD hamsters and stimulated with LPS produced significantly higher levels of TNFα compared to similarly challenged macrophages cultured from SD hamsters ($F_{2,37}=20.641$, $p<0.0001$). Macrophages cultured with a control treatment (DMEM only) from LD and SD hamsters produced similar low levels of TNFα and were thus pooled for all analyses. Control-treated macrophages produced significantly less TNFα compared to LPS-stimulated

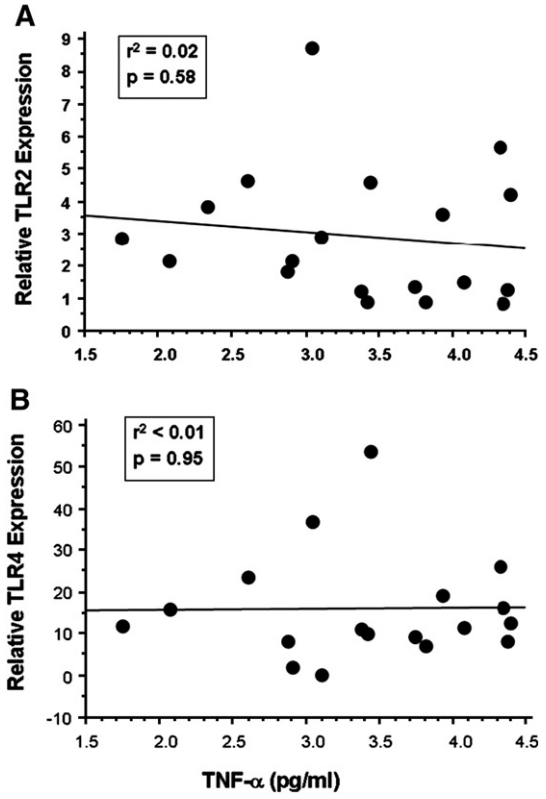

Fig. 4. Relationship between (A) relative TLR2 expression or (B) relative TLR4 expression in elicited peritoneal macrophages and production of TNFα after stimulation with LPS by elicited peritoneal macrophages from the same individual. Regression analyses were similarly non-significant when examined separately for each day length treatment, so treatments were collapsed for this depiction. Macrophages were collected from individuals maintained in either LD (16 h light:8 h dark) or SD (8 h light:16 h dark) conditions for 11 weeks.

macrophages cultured from both LD and SD hamsters (Fig. 2, LD-Control: $p<0.0001$, SD-Control: $p=0.008$).

3.3. TLR2 and TLR4 expression in elicited peritoneal macrophages

TLR2 and TLR4 expression in elicited peritoneal macrophages (Fig. 3, TLR2: $F_{1,20}=2.67$, $p=0.12$, TLR4: $F_{1,19}=0.049$, $p=0.83$) was statistically similar in cells/tissues collected from LD and SD animals. Additionally, there was no relationship between relative expression of TLR2 or TLR4 in macrophages and the amount of TNFα those macrophages produced (Fig. 4, TLR2: $R^2=0.018$, $p=0.58$, TLR4: $R^2<0.01$, $p=0.95$). When animals in each photoperiod treatment were examined separately, similar results were obtained, with no significant relationship between expression of TLR2 or TLR4 and the amount of TNFα those macrophages produced (LD: TLR2, $R^2=0.027$, $p=0.65$, TLR4, $R^2=0.012$, $p=0.76$, SD: TLR2, $R^2=0.07$, $p=0.51$, TLR4, $R^2=0.07$, $p=0.52$).

4. Discussion

We hypothesized that macrophages collected from Siberian hamsters maintained in short, winter-like day lengths (SD) would exhibit significantly lower relative expression of TLR2 and TLR4 than macrophages collected from hamsters in long, summer-like day lengths, because TLR4 and TLR2 are the macrophage PRRs responsible for the recognition of and response to LPS (Lien et al., 2000). Additionally, we hypothesized that photoperiod-driven differences in expression of these Toll-like receptors would be directly related to a decrease in TNFα production in SD animals following stimulation with LPS in culture. These hypotheses were not supported by the results of our experiment.

As previously documented, SD hamsters exhibited phenotypic changes compared to LD animals (Hoffman, 1973). Body mass, epididymal adipose tissue mass, and paired testes mass were all significantly decreased after 11 weeks of SD exposure. When macrophages taken from hamsters were challenged with LPS, cells from SD and LD hamsters produced measurable amounts of TNFα, but macrophages taken from SD hamsters produced significantly less compared to cells from LD hamsters. Our results are similar to those reported previously (Prendergast et al., 2003). Thus, short-day lengths inhibit the abilities of macrophages to respond to a potential pathogen. These results are consistent with other studies documenting inhibition of macrophage-mediated phagocytosis (Yellon et al., 1999), production of oxidative molecules (Yellon et al., 1999), and fever (Bilbo et al., 2002) by exposure to winter-like day lengths and melatonin treatments. Taken together, it seems likely that many of the measured seasonal differences in immune parameters, including those resulting from T and B-lymphocyte function, may be directly or indirectly mediated by changes in macrophage function, through altered production of important pro-inflammatory cytokines.

No difference, however, was observed in expression of either TLR2 or TLR4 between LD and SD peritoneal macrophages, despite the photoperiod-based modulation of cytokine production by those macrophages. We predicted photoperiod-driven differences in expression of TLR4 in elicited peritoneal macrophages because those macrophages responded to LPS differently in SD vs. LD conditions, and TLR4 is the receptor known to most effectively recognize and respond to LPS (Aderem and Ulevitch, 2000). Instead, TLR4 was expressed at similar levels in SD and LD animals, and expression of TLR4 in macrophages was not related to the amount of TNFα produced by those macrophages. TLR2, which has been shown to be upregulated in response to LPS in vivo, was also not expressed differently in LD vs. SD animals. This is not altogether unexpected because some studies suggest that TLR2 is of limited importance in response to LPS (Lien et al., 2000). Failure to detect valid differences in relative expression of TLR2 and TLR4 in macrophages is unlikely because photoperiod causes a significant 38% difference in TLR2 expression in splenic tissue (K. Navara, unpublished data), and post hoc power analyses determined that there was an 84% chance of detecting a similar difference in the current analyses involving macrophages (K. Navara, unpublished data). It is possible that we did not see differences in TLR2 and TLR4 expression because the macrophages were collected and elicited through stimulation with thioglycollate. It has been shown previously that thioglycollate-elicited peritoneal macrophages are phenotypically different from resident or inactive macrophages (Bredt and Snyder, 1994). It is

possible that expression levels of inactive macrophages may vary according to day length while levels in activated macrophages do not. In the current study, however, we document that elicited and thus activated macrophages respond differently to LPS when in LD conditions by producing significantly more TNFα. If differences in expression of TLR2, TLR4, or both TLRs were responsible for the differences in macrophage responsiveness we document between photoperiod treatments, then we expect that we would have detected differences in expression levels between the treatment groups, which we did not. Thus, these data suggest that photoperiodic modulation of macrophage function may not be occurring through regulation of TLR2 or TLR4 mRNA expression, and that another target of photoperiod-based modulation in hamster peritoneal macrophages likely exists.

Although overall TLR2 and TLR4 expression do not differ between peritoneal macrophages in the two photoperiodic treatments, it is possible that the abilities of these receptors to bind to their ligands and/or trigger downstream effects may differ. For example, a major downstream target involved in macrophage functions, nuclear factor kappa B (NFκB), has been shown to be inhibited by melatonin treatment, the hormone responsible for transducing day length into a physiological signal (Gilad et al., 1998; Yoshimura et al., 1999; Eicher et al., 2004). Thus, future experiments examining TLR2 and TLR4 function in these cells in relation to day length would be helpful. This and future molecular studies are crucial to a full understanding of the vertebrate immune system, as well as variation in immunological mechanisms among species and in response to environmental conditions. With the rapid increase in the identification of non-traditional vertebrate genomes, it will become possible and increasingly important to integrate molecular approaches into the study of ecoimmunology.

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