Circadian clock regulates the host response to *Salmonella*

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Edited by Joseph S. Takahashi, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, and approved April 18, 2013 (received for review December 15, 2011)

Organisms adapt to day–night cycles through highly specialized circadian machinery, whose molecular components anticipate and drive changes in organism behavior and metabolism. Although many effectors of the immune system are known to follow daily oscillations, the role of the circadian clock in the immune response to acute infections is not understood. Here we show that the circadian clock modulates the inflammatory response during acute infection with the pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Mice infected with *S.* Typhimurium were colonized to higher levels and developed a higher proinflammatory response during the early rest period for mice, compared with other times of the day. We also demonstrate that a functional clock is required for optimal *S.* Typhimurium colonization and maximal induction of several proinflammatory genes. These findings point to a clock-regulated mechanism of activation of the immune response against an enteric pathogen and may suggest potential therapeutic strategies for chronopharmacologic interventions.

clock genes | inflammation | gastroenteritis | intestine | microbes

The circadian (from the Latin *circa diem*) system consists of an internal clock, whose molecular components drive changes in organism behavior, metabolism, and immune system, thus contributing to body homeostasis (1). Many immune functions follow a circadian rhythm, including expression of several cytokines, the number of lymphocytes, and activity of phagocytic cells (2–4). In addition, a variety of chronic inflammatory diseases, from rheumatoid arthritis to obesity, are linked to altered circadian regulation (5–7), and clock disruption is associated with dysregulation of the inflammatory response (8). These studies underline the importance of circadian rhythm in immune functions and inflammation but do not document its role in regulating the host response to infection.

The pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most common causes of foodborne illness worldwide (9). Acute infection in humans is characterized by a massive intestinal inflammation with neutrophil migration to the gut within 48 h after ingestion of contaminated food or water. This infection can be modeled in mice pretreated with streptomycin 24 h before infection (colitis model) (10–13). Initiation of the host response requires virulence factors, including two type III secretion systems (T3SS-1 and -2), which mediate *S.* Typhimurium invasion of the intestinal mucosa and promote its replication within antigen-presenting cells. Other bacterial components, such as lipopolysaccharide (LPS) and flagellin, activate Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in macrophages and/or epithelial cells, resulting in the secretion of proinflammatory cytokines that ultimately recruit neutrophils and induce expression of antimicrobial proteins (14). Hence, the initial interactions of host cells with secreted effector proteins, LPS and flagellin, are key events in the initiation of the host response to *S.* Typhimurium. It is known that mice are susceptible to LPS-induced endotoxic shock as well as to TNF-α toxicity, depending upon the administration time during the 24-h light–dark (LD) cycle (15, 16). Furthermore, direct molecular links between the circadian and innate immune systems have been recently established (17, 18). Nonetheless, whether the circadian clock also influences the host response to a pathogen is not known. Here we used the mouse colitis model to determine how the circadian clock regulates the host response during acute *Salmonella* infection.

Results and Discussion

Differential Day–Night Response to *Salmonella* Infection. To determine whether the circadian clock regulates the host response to infection with *S.* Typhimurium, we infected wild-type (WT) mice by oral gavage either at 10:00 AM (day, early rest phase; zeitgeber time 4, ZT4) or at 10:00 PM (night, early active phase; ZT16). At 48, 60, 72, and 78 h postinfection (p.i.), mice were killed, and tissue samples collected for bacteriology, histopathology, and gene expression analyses (Fig. 1). We observed that *S.* Typhimurium colonization significantly changed with time of infection, especially at later time points. Notably, *S.* Typhimurium numbers were significantly increased in the colon content of mice infected at 10:00 AM in comparison with 10:00 PM at both 72 and 78 h p.i. (Fig. 1*A*); colonization of Peyer's patches and spleen was also significantly higher in mice infected during the day (Fig. S1*A*). Next, we determined whether the degree of the host response to infection changed with the time of inoculation. Histopathology showed that ceca from mice infected during the day were on average more inflamed than those from mice infected during the night, at 48, 72, and 78 h p.i. (Fig. 1 *B* and *C*; Figs. S1*B* and S2). In contrast, at 60 h p.i., a mild increase in inflammation was observed in mice infected at night, suggesting that the time of infection was not the only variable influencing the inflammatory response. Reduced signs of cecal inflammation were characterized by low-grade submucosal edema and neutrophil influx (Fig. 1 *B* and *C* and Figs. S1*B* and S2). Major differences were also found in the levels

Author contributions: M.M.B., E.D., B.G., M.R., and P.S.-C. designed research; M.M.B., E.D., J.Z.L., B.G., C.B., M.Z., R.A.E., S.S., and M.D.G. performed research; S.D. contributed new reagents/analytic tools; M.M.B., E.D., J.Z.L., B.G., M.Z., R.A.E., P.B., M.D.G., M.R., and P.S.-C. analyzed data; and M.M.B., E.D., M.R., and P.S.-C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE46356). The complete representation of our computational modeling approach to predict the transcriptional regulatory networks involved in the control of genes in each of the four clusters identified in our genomic profiling analysis is available at www.ics.uci.edu/~baldig/CLOCK/salmonella.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1120636110/-/DCSupplemental.

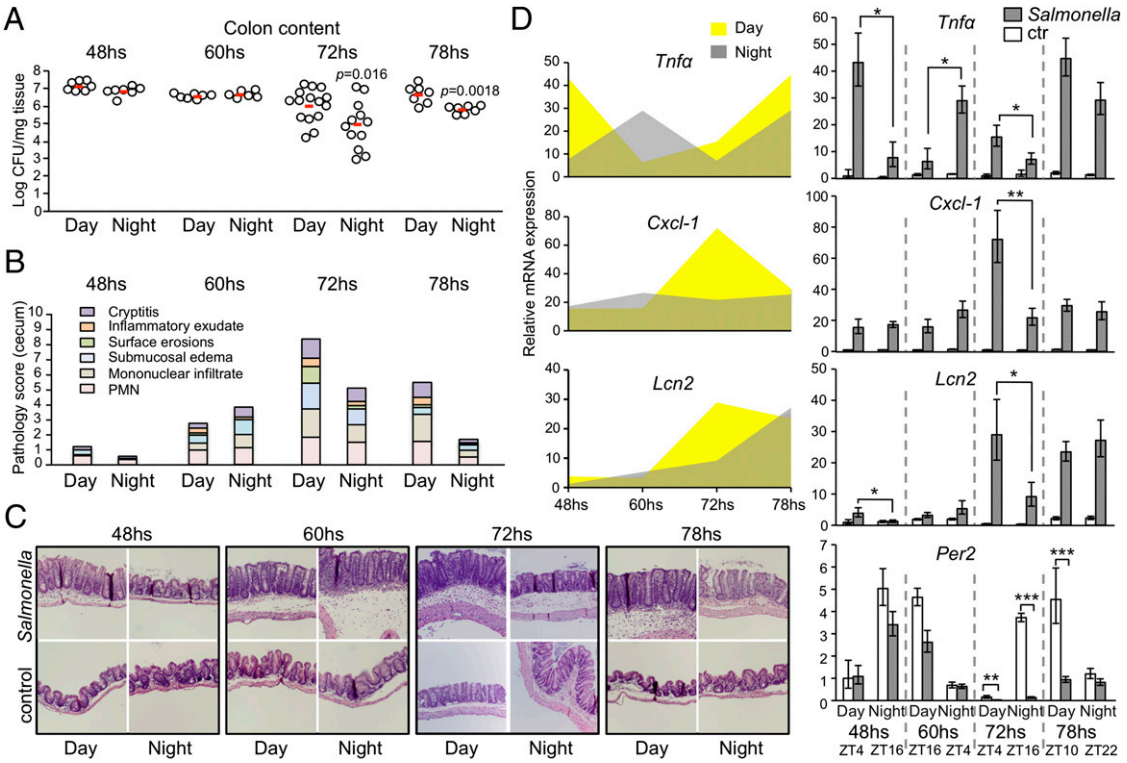

Fig. 1. The inflammatory response is time-of-day–dependent in the cecum of mice infected with *S.* Typhimurium. (*A*) Recovery of *S.* Typhimurium from colon content of WT mice at 48, 60, 72, and 78 h p.i. at different circadian times (day, 10:00 AM or ZT4; night, 10:00 PM or ZT16). Each circle represents an individual animal. Red bars indicate the geometric means ($n \geq 7$). (*B*) Cecal histopathology score of mice from *A*. Each bar represent the combined score of at least seven mice. PMN, polymorphonuclear leukocytes. (*C*) Representative images (10× magnification) of cecal inflammation in WT mice infected (*Salmonella*) or not at day (ZT4) or night (ZT16). (*D*) mRNA expression of *Tnfα*, *Cxcl-1*, *Lcn2*, and *Per2* in the cecum of mice from *A*–*C* ($n \geq 7$). Data are represented as geometric means of fold increases compared with uninfected WT (48 h) day ± SEM. Significant day–night changes are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (*Left*) Graphs with yellow and gray areas represent the progression of mRNA expression of *Tnfα*, *Cxcl-1*, and *Lcn2* depending on the time of infection.

of cryptitis and surface erosions (Fig. S1*B*), which constitute other classical signs of inflammation (10–12). To ascertain whether these differences were accompanied by changes in gene expression, we analyzed expression of proinflammatory cytokine *Tnfα*, neutrophil chemoattractant chemokine (C-X-C motif) ligand 1 (*Cxcl-1*), and antimicrobial peptide lipocalin-2 (*Lcn-2*) in the cecum of infected and uninfected mice and found significant differences during the course of infection (Fig. 1*D* and Table S1). Consistent with our previous findings (11–13), mice infected during the day showed increased expression of these genes compared with uninfected controls, and the expression was dependent both on time of infection and time of death (Fig. 1*D*). Genes in mice infected in the morning (ZT4) or at night (ZT16) showed maximal expression when killed during the day (48 and 72 h for mice infected at ZT4; 60 h for mice infected at ZT16). This finding was particularly notable for *Tnfα* because its expression was increased at both 48 and 72 h in mice infected at ZT4 (ZT4 death) relative to those infected at ZT16 (ZT16 death), and the opposite trend was observed at 60 h (ZT16 death for mice infected at ZT4; ZT4 for mice infected at ZT16). In line with this observation is the absence of significant changes in expression in mice killed 78 h after infection, corresponding to ZT10 for mice infected at ZT4 and ZT22 for mice infected at ZT16 (Fig. 1*D*). Additionally, the overall expression levels of the three proinflammatory genes analyzed were reduced in mice infected at ZT16 (yellow and gray graphs of Fig. 1*D*), consistently with the differences observed in colonization level and pathology score.

To establish whether circadian transcription follows a normal profile during infection we analyzed the expression of the clock gene period 2 (*Per2*). *Per2* oscillation in uninfected mice demonstrated proper synchronization (Fig. 1*D*). Notably, *Per2* expression was progressively down-regulated after infection (Fig. 1*D*).

These results suggest that a circadian mechanism may regulate components of the innate immune response to acute *Salmonella* infection, also revealing a profound repression of circadian components during infection.

***Clock* Mutation Affects Cytokine Production from LPS-Challenged and *Salmonella*-Infected Bone Marrow-Derived Macrophages.** Our finding that the inflammatory response to *S.* Typhimurium infection is time-of-day–dependent in vivo prompted us to determine whether we could reproduce similar results in vitro in a less complex system. As detailed earlier, macrophages are involved in the first response to *S.* Typhimurium infection. Moreover, macrophages have an efficient clock machinery, and LPS-activated pathways in these cells are under tight circadian regulation at multiple levels (3, 4). However, bone marrow-derived macrophages (BMDMs) do not synchronously oscillate after 1 wk of differentiation in vitro (Fig. S3*A*), and LPS stimulation at different circadian times leads to similar levels of *Il-6* expression in these asynchronous cultures (Fig. S3*B*). We therefore tested whether BMDMs could be synchronized by common methods such as dexamethasone or high-serum treatments (Fig. S3 *C* and *D*). Oscillation of circadian genes *Per2* (Fig. S3 *C* and *D*), cryptochrome 1 (*Cry1*) and brain and muscle ARNT-like protein 1 (*Bmal1*) (Fig. S3*C*) confirmed entrainment of these cells. To better evaluate the contribution of the circadian system to the expression of proinflammatory genes, we added LPS (1 μg/mL) to synchronized macrophages at different times of their circadian cycle and followed the expression profile of *Il-6* (Fig. S3*D*). As expected, we obtained different curves of expression depending on the time of LPS administration, with minimal induction for administration at T18 or T30 and major induction at T12 or T24, where T0 is the time when synchronization began. Notably,

expression levels appear to be dependent both on the time of treatment and on the time of collection, as suggested by our in vivo data.

We next ascertained whether disruption of the circadian clock could affect the function of some central component of the LPS response. Circadian gene expression in mice carrying a mutation of the master circadian regulator *Clock* [*Clock* mutant or clock/clock mice (19)] is both phase-shifted by 8 h as well as reduced compared with WT littermates (20). *Clock* mutant mice also fail to rhythmically express a number of immunoregulatory genes in the liver (21). Therefore, we followed the timing of expression of different cytokines after LPS stimulation of BMDMs isolated from either WT or *Clock* mutant mice (Fig. 2). Remarkably, we observed an overall reduction in the fold induction of the proinflammatory genes *Il-6*, *Il-1β*, *Tnfα*, *Cxcl-1*, *Ifn*-β, and Chemokine (C-C motif) ligand 2 (*Ccl2*) in response to LPS stimulation in BMDMs isolated from *Clock* mutant mice compared with WT mice (Fig. 2*A*); BMDMs isolated from *Clock* mutant mice also exhibited reduced secretion of IL-6 and TNF-α after 24 h of LPS stimulation (Fig. 2*B*). A comparable reduction in the levels of IL-6 in the supernatant of BMDMs isolated from *Clock* mutant mice, compared with WT mice, was obtained after infection with *S.* Typhimurium (Fig. 2*C*). Because the induction of IL-6 is largely dependent on LPS stimulation of the TLR4 pathway, we also infected BMDMs with an isogenic *S.* Typhimurium strain carrying a mutation in the lipid A acylation pathway (*msbB* mutant), which impairs signaling through TLR4 (11). As predicted, secretion of IL-6 was reduced in BMDMs infected with the *msbB* mutant compared with infection with WT *S.* Typhimurium. In contrast, both *S.* Typhimurium WT and the *msbB* mutant elicited similar low levels of secretion of IL-6 (Fig. 2*C*) in BMDMs isolated from *Clock* mutant mice, indicating that TLR4 signaling in response to *S.* Typhimurium was not induced in the absence of a functional clock. Next we sought to investigate whether the secretion of the proinflammatory cytokine IL-1β was also impaired in *Clock* mutant mice. IL-1β is directly induced by TNF-α, causing its expression to peak later than *Tnfα*, and mature IL-1β secretion requires both TLR4 and NLR signaling (22). Upon *S.* Typhimurium infection of BMDMs, LPS activation of TLR4 induces pro–IL-1β expression, which is followed by NLR-mediated signaling through ice protease-activating factor/NLR family CARD domain-containing protein 4 (Ipaf/Nlrc4) in response to *Salmonella* T3SS-1 secretion of flagellin into the cytosol (22). As expected from these earlier studies, *S.* Typhimurium strains carrying either a mutation in *msbB* (unable to signal through TLR4), a mutation in both flagellin genes (*fliC fljB* mutant, unable to signal through Ipaf/Nlrc4), or a mutation in T3SS-1 (*invA* mutant, unable to signal through Ipaf/Nlrc4) induced secretion of lower levels of IL-1β compared with *S.* Typhimurium WT (Fig. 2*D*) in BMDMs from WT mice. In contrast, infection of BMDMs from *Clock* mutant mice with *S.* Typhimurium WT resulted in a marked reduction of IL-1β secretion. Moreover, all *S.* Typhimurium mutants elicited similar low levels of IL-1β in *Clock* mutant BMDMs. Collectively, we observed that secretion of proinflammatory cytokines in *Clock* mutant BMDMs was particularly low and comparable to levels elicited by *S.* Typhimurium mutants designed to evade activation of particular components of the inflammatory response. Thus, an intact circadian machinery appears to be required for the induction of proinflammatory cytokines in vitro in response to *Salmonella* infection.

Alteration of Time-Dependent Response to *Salmonella* in *Clock* Mutant Mice. Because the circadian clock is necessary for a robust proinflammatory response in isolated macrophages, we explored its involvement in the host response to *Salmonella* in vivo. We infected *Clock* mutant mice at 10:00 AM (ZT4) or 10:00 PM (ZT16) (Fig. 3*A*). Differently from WT mice, similar numbers of *S.* Typhimurium were recovered 72 h p.i. (Fig. 3*A*). Similar results were also obtained when *Clock*-deficient mice ($Clock^{-/-}$) and their WT littermates were infected, thus confirming that a functional CLOCK protein is necessary for the observed effect. (Fig. S4*A*). Furthermore, a significant difference was found in the colonization of Peyer's patches between WT mice infected at day and *Clock* mutant mice, even if these lymphatic structures were significantly enlarged in *Clock* mutant mice during infection (Fig. S4*B*). Moreover, the histopathology revealed higher inflammation in *Clock* mutant mice infected at night compared with mice infected during the day (Fig. 3 *B* and *C*; Fig. S4 *C* and *D*). Altogether, these results suggest that a circadian mechanism regulates components of the host response upon *Salmonella* infection and that clock disruption largely affects this response.

Transcriptome of Cecum After *Salmonella* Infection. Because previous microarray studies revealed that ∼10% of genes follow circadian oscillation in almost all tissues (7), we set out to gain further insight into the circadian clock regulation of the host response to infection by analyzing global changes in gene expression by microarray analysis. To determine whether circadian regulation occurs at the transcriptional level in response to infection in vivo, we analyzed the differential gene expression profile by microarray analysis of the cecum in WT and *Clock* mutant mice 72 h p.i. with *S.* Typhimurium or mock control at different circadian times (day, ZT4; night, ZT16). Our analysis identified four main clusters of transcripts with different patterns of expression, which we

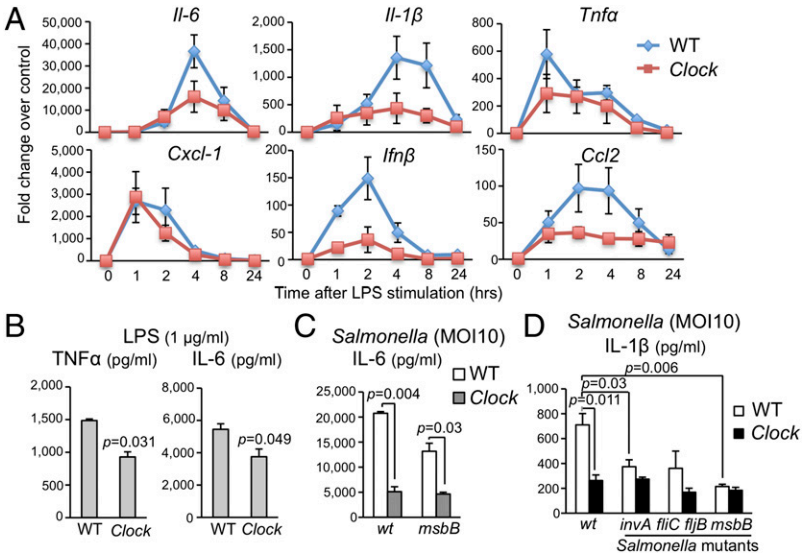

Fig. 2. Reduced cytokine production from macrophages of *Clock* mutant mice. (*A*) Time course of mRNA expression of different cytokines after LPS stimulation of BMDMs. Time 0 (unstimulated cells) in both WT and *Clock* mutant cells was set to 1. Bars represent mean ± SEM ($n = 3$). (*B*) Supernatant protein level of TNF-α and IL-6 from BMDMs of WT and *Clock* mutant mice after 24 h of LPS stimulation. Bars represent means ± SEM ($n = 4$). (*C*) IL-6 production in BMDMs from WT and *Clock* mutant mice 24 h p.i. with *S.* Typhimurium WT or with the *msbB* mutant. MOI, multiplicity of infection. (*D*) BMDMs from WT and *Clock* mutant mice were infected with different strains of *S.* Typhimurium as indicated. After 24 h, supernatants were collected, and secretion of IL-1β was measured. Data represent means ± SEM ($n = 3$).

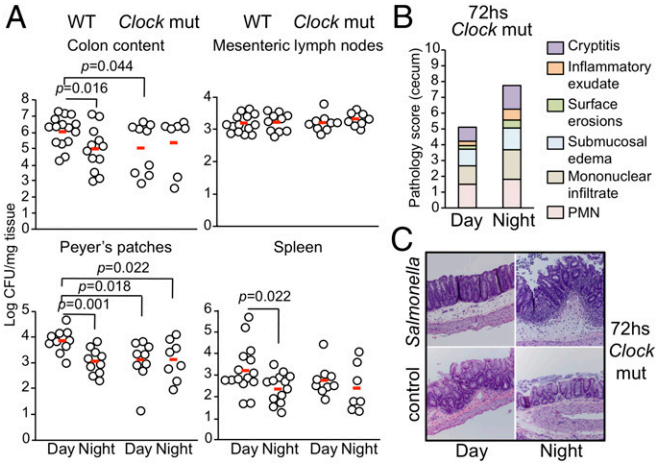

Fig. 3. Altered inflammatory response in the intestine of *Clock* mutant mice after in vivo infection with *S.* Typhimurium. (*A*) Tissue colonization in WT and *Clock* mutant mice 72 h p.i. with *S.* Typhimurium. Day, 10:00 AM, ZT4; night, 10:00 PM, ZT16. Each circle represents an individual animal. Red bars indicate the geometric means ($n \geq 8$). WT mice are as in Fig. 1*A* and Fig. S1*A*. (*B*) Histopathology of cecum of infected *Clock* mutant mice from *A*. Each bar represent the combined score of at least eight mice. PMN, polymorphonuclear leukocytes. (*C*) Representative images (10× magnification) of the ceca from *Clock* mutant mice at 72 h p.i. (*Salmonella*) or mock, at day or night.

numbered clusters 1–4 (Fig. 4*A* and Dataset S1). The gene expression profile in cluster 1 was characterized by the induction of genes linked to inflammation and immunity in response to infection, including genes involved in immune cell activation and antimicrobial responses. Genes in cluster 2, related to glycolipid biosynthesis and protein transport, were up-regulated during infection. In contrast, genes involved in lipid and steroid metabolism (cluster 3) and dendritic cell maturation (cluster 4) were mostly down-regulated in response to infection.

Notably, several genes in all four clusters were regulated in a time-specific and clock-dependent manner during infection (Fig. 4*A*, Datasets S2, S3, and S4). Moreover, a detailed pathway analysis identified several transcripts with a different day–night expression in WT mice (Datasets S1 and S2). Specifically, we observed the most relevant changes of gene expression for transcripts involved in the antimicrobial response (Fig. S5*A*), acute inflammation (Fig. S5*B*), leukocyte chemotaxis, and antigen presentation and processing (Fig. 4*A* and Dataset S1). Consistent with our observations, the analysis of genes in cluster 1 revealed a general low level of induction of the inflammatory response in *Clock* mutant mice, a response that was slightly higher at night (Fig. 4*A*, Fig. S5 *A* and *B*, and Dataset S3). These observations were further validated through analysis of a subset of cluster 1 genes by quantitative real-time PCR (Fig. 4 *B* and *C* and Table S1). In particular, many of the proinflammatory (*Tnfα*, *Il-17*, *Cxcl-1*) and antimicrobial [*Lcn2*, regenerating islet-derived 3 γ and β (*Reg3γ and β*)] genes analyzed were up-regulated at night upon infection, but to lower levels than during the day. In contrast, levels of expression in *Clock* mutant mice were mostly similar between day and night, with only a couple genes [*Tnfα* and S100 calcium binding protein A8 (*S100a8*)] that were expressed at higher levels at night than during the day.

Because expression of different cytokines follows a circadian rhythm (2–4), we investigated whether intrinsic temporal changes may influence gene expression in the intestine of both WT and *Clock* mutant mice (Fig. S6*A* and Dataset S5). As expected, a higher proportion of genes oscillated in the cecum from WT uninfected mice compared with *Clock* mutant mice (Fig. S6 *A* and *B*), including circadian genes, metabolic genes, and genes involved in the immune response (Fig. S6 *A* and *C* and Dataset S5). Because we observed that some transcripts (*Tnfα*, *Cxcl-1*, and *Lcn2*) did not show differences between day and night, whereas other genes (*Il-17*, *Reg3β*, and *Reg3γ*) were strongly up-regulated at night (Fig. 4*C*), we sought to determine the absolute expression levels of each gene (Fig. S7). This analysis showed that the expression level of the majority of genes analyzed was lower in *Clock* mutant mice in both basal condition and after infection (Fig. S7). Together, these results suggest that circadian regulation influences cytokine expression at different levels, by modifying both basal and induced transcription of pro- and anti-inflammatory genes during acute infection.

Additional and Uncharacterized Circadian Regulations. Little is known about the metabolic and physiological changes that occur during the circadian cycle in infected mice. Our data suggest that the rhythmicity of circadian and metabolic genes is suppressed or attenuated during infection (Fig.4*A* and Dataset S1). Furthermore, we noticed that genes peaking at different circadian times also undergo opposite transcriptional regulation after infection (Fig. 4*A*). For instance, cluster 2 genes massively oscillate in basal conditions and are strongly induced after infection in WT mice; however, their cycle is inverted and their induction is completely abolished in *Clock* mutant mice. Similarly, clusters 3 and 4 contain genes that are up-regulated at basal levels at day or night, respectively, but are repressed following infection (Fig. 4*A*). Within these categories are the clock genes (Dataset S1 and Fig. S8). Consistent with previous studies (23), circadian components [*Bmal1*, also called Aryl hydrocarbon receptor nuclear traslocator-like (*Arntl*), period proteins, cryptochromes, neuronal PAS domain protein 2 (*Npas2*), nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*)] oscillated in uninfected mice and showed either a marked reduction of the oscillation or a phase-shifted rhythm in *Clock* mutant mice (Fig. S8 *A* and *B*). Also, genes of the main metabolic pathways were found in these clusters, as predicted by previous studies (24). Moreover, the time-dependent changes observed in the transcription of genes involved in dendritic cell development and leukocyte function, as well as other immune genes included in cluster 4 (Fig. 4*A* and Dataset S1), suggest a possible circadian control of the development of innate and adaptive immunity following infection.

Computational Analysis Reveals Connections Between Circadian Transcription and Inflammatory Response. To extend our comprehension of the transcriptional pathways participating in the circadian activation of the host defense against infection, we used a computational modeling approach to predict the transcriptional regulatory networks involved in the control of genes in each of the four clusters identified in our genomic profiling analysis (the complete representation is available at www.ics.uci.edu/~baldig/CLOCK/salmonella/). In cluster 1, we identified main synergistic nodes connecting transcription driven by BMAL1:CLOCK (ARNTL:CLOCK) to critical inflammatory pathways (Fig. 4*D* and Fig. S9*A*). The gene node graphics show transcription factors with significant changes in expression between different conditions (blue, WT infected vs. uninfected day; green, WT infected vs. uninfected night; brown, *Clock* mutant infected vs. uninfected day; orange, *Clock* mutant infected vs. uninfected night; $P < 0.05$; Fig. 4*D* and Fig. S9 *A* and *B*). In agreement with previous reports, NF-κB and hypoxia inducible factor 1, alpha subunit (HIF-1α) are the transcription factors with the largest numbers of connections (25, 26). Both NF-κB and HIF-1α share many target genes with the transcription factor BMAL1 (ARNTL), which cooperates with CLOCK in regulating circadian transcription. Notably, HIF-1α appeared to mediate significant changes in all four conditions that we analyzed. Nodes were much larger in WT mice compared with *Clock* mutant mice, thus indicating that many pathways regulated by HIF-1α also require a functional clock system. This observation is of particular interest because cross-talk between hypoxic and circadian pathways has been proposed, and *Hif-1α* is thought to be a clock-controlled gene (27).

Circadian Regulation of Antimicrobial Peptides Influences *Salmonella* Growth. We next sought to determine whether circadian changes influence *Salmonella* growth, which is known to be enhanced by intestinal inflammation, in part because *Salmonella* is resistant to some antimicrobial proteins secreted in the inflamed gut (11). As shown in Fig. 1*D* and Fig. 4*B* and Figs. S5*A* and S7, the gene *Lcn2*, which encodes for lipocalin-2, an antimicrobial peptide that

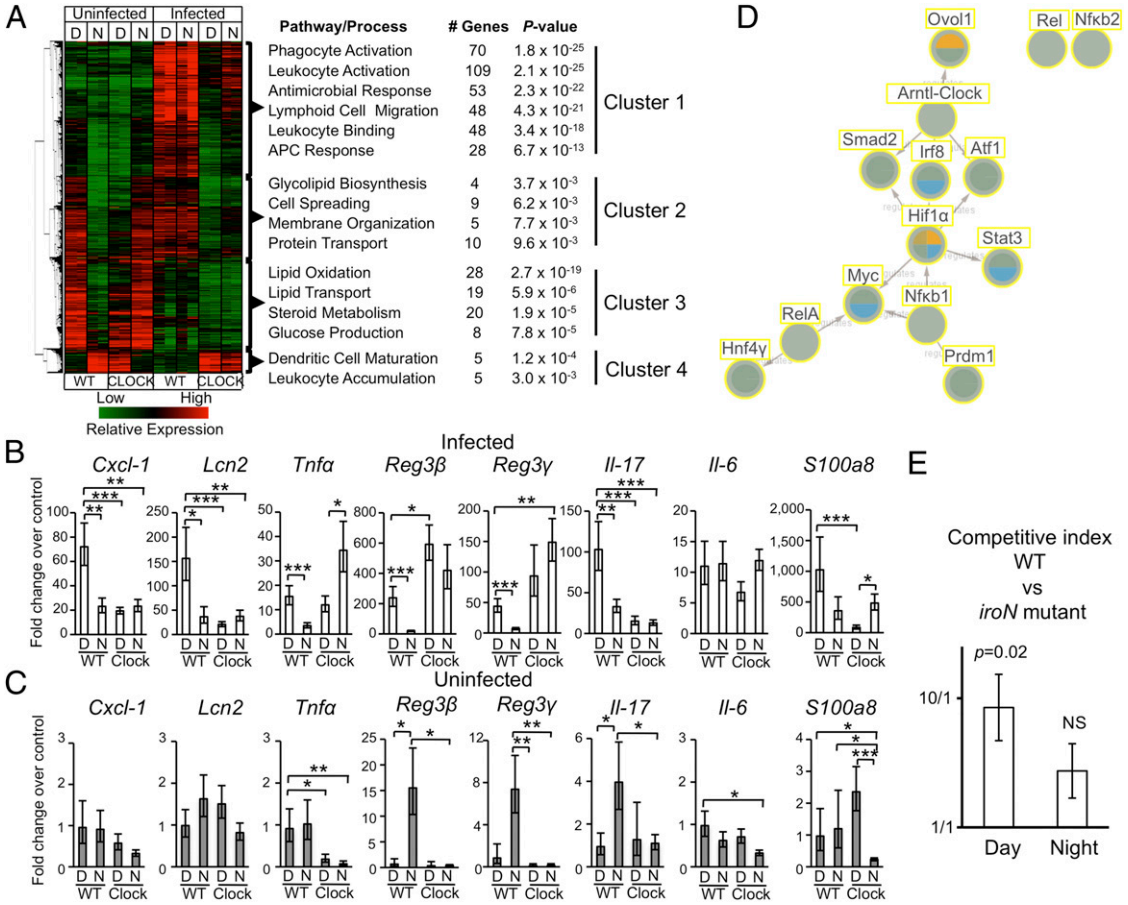

Fig. 4. Microarray analysis from cecum of mice infected with *S.* Typhimurium reveals a circadian mechanism modulating the response to acute bacterial infection. (*A*) Heat diagram showing changes in gene expression detected in the ceca of mice 72 h p.i. with *S.* Typhimurium at day (D, ZT4) or night (N, ZT16) in WT and *Clock* mutant mice, compared with uninfected controls ($n \geq 3$). Representative results from two animals are shown. Relative increase (red) or decrease (green) of mRNA level is shown. A list of the most represented subcategories of genes from each cluster, the number of genes included in each subcategory, and the relative *P* value are shown. (*B* and *C*) Transcriptional profiles of selected proinflammatory/antimicrobial genes identified in cluster 1. Significant changes are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*D*) Network of transcription factors involved in regulation of subsets of genes included in cluster 1. Significant changes ($P < 0.05$) are shown as colored circles (blue, WT infected vs. uninfected day; green, WT infected vs. uninfected night; brown, *Clock* mutant infected vs. uninfected day; orange, *Clock* mutant infected vs. uninfected night). (*E*) Competitive infection with a mixture of *S.* Typhimurium WT and *iroN* mutant at two different times of the day (day, ZT4; night, ZT16). Bars indicate the average competitive index of bacteria recovered from colon contents. Data represent geometric means ± SEM ($n = 10$ each group).

sequesters the essential nutrient iron and thereby reduces the growth of susceptible bacteria (28), has a different day–night expression following infection. *S.* Typhimurium is resistant to LCN2 because it encodes for the synthesis and uptake of a high-affinity iron chelator, termed salmochelin (29). High expression of LCN2 in the inflamed gut enhances *Salmonella* colonization over competing microbes, whereas a LCN2–sensitive mutant in the salmochelin receptor IroN has a colonization defect and is outgrown by *Salmonella* WT in mice expressing *Lcn2*, but not in mice carrying a *Lcn2* mutation (11). Thus, we hypothesized that WT *S.* Typhimurium would have a greater colonization advantage in competition with the *iroN* mutant during the day, when *Lcn2* is highly induced, whereas we would recover both strains at similar levels at night, when *Lcn2* is expressed at lower levels. Infection of WT mice at ZT4 or ZT16 with a 1:1 mixture of WT *S.* Typhimurium and the *iroN* mutant confirmed this prediction. *Salmonella* WT outcompeted the *iroN* mutant in the colon content collected 72 h p.i. from mice infected during the day (Fig. 4*E*). In contrast, *S.* Typhimurium WT had no significant advantage over the *iroN* mutant at night (Fig. 4*E*), when *Lcn2* was induced at lower levels. These results further suggest that circadian expression of *Lcn2*, and possibly other antimicrobial peptides, influences *Salmonella* colonization and its competitive advantage over susceptible microbes.

Conclusions

Although in lower organisms a direct connection between the circadian system and susceptibility to infection has been established (30–31), this link has remained elusive in mammals. Here we show that the host response to *Salmonella* infection changes between day and night, corresponding to rest and active phase in mice, and that this differential response is driven by the circadian clock. Immediately after infection, the inflammatory response increases by following an oscillatory circadian course, with higher response during the day and reduction at night. This effect is a consequence of the clock-controlled expression of many genes whose products play a fundamental role in host defense against infection. Among these, genes encoding antimicrobial peptides display a robust circadian oscillation during infection, resulting in the modulation of *Salmonella* colonization and its competition with susceptible microorganisms for a niche in the inflamed gut. Although *Salmonella* is resistant to several antimicrobial responses, including LCN2 and REG3γ, commensal microbes are more susceptible. Therefore, circadian regulation of antimicrobial proteins may be important to control the overgrowth of the microbiota and prevent infection from

susceptible microorganisms. Previous reports have shed light on the effect that ablation of clock genes has on specific immune parameters (32). In flies, two circadian mutants are more sensitive to some bacterial pathogens than WT organisms (33). Furthermore, *Per2*-deficient mice are more resistant to LPS-induced endotoxic shock than WT mice (34), and disruption of the circadian clockwork by targeting *Bmal1* or *Nr1d1* removed the circadian gating of the endotoxin-induced cytokine response, both in vitro and in vivo (17). Our results show that *Clock* mutant mice have an altered timing of reaction following *Salmonella* infection, as well as a significant reduction in the expression of proinflammatory genes. Although a circadian clock-independent function of clock genes cannot be formally excluded, recent findings also suggest that alteration of circadian gene expression is likely to be responsible for the phenotype of *Clock* mutant mice (17, 35).

In our experiments, rhythmicity of circadian and metabolic genes is suppressed or attenuated at the site of infection, supporting the concept of intimate bidirectional communication between the circadian and immune systems (36, 37). Our hypothesis is that immune factors contribute to the daily coordination of the circadian system, but powerful immunological challenges send signals to disrupt this regulation, possibly by uncoupling some of the circadian outputs, leading to reduction of the amplitude of the oscillations. Conversely, the circadian system dictates the right timing for the host response to infection by regulating components of the immune system both at the level of individual cells and at the systemic level, through autonomic and endocrine outputs (18). The circadian system modulates the activity of several transcription factors that are important regulators of immune functions, including HIF1-α, STAT1, STAT3, and NF-κB (38–40). We were able to confirm that these factors, together with many more, create the main transcriptional regulatory networks during infection in our genomic profiling analysis. The results of our analysis point to connections of the circadian clock to other functional systems, including metabolic and immune, during infection and will be instrumental for future studies focused on elucidating these mechanisms.

Materials and Methods

Mouse Experiments. Mice kept in 12:12 light:dark conditions were pretreated with streptomycin (0.1 mL of a 200 mg/mL solution in sterile water) intragastrically, as described (10–12) 24 h before inoculation with WT *S.* Typhimurium [100 μL containing ~1×10^9 colony-forming units (cfu)] or with a 1:1 mixture of *S.* Typhimurium WT and *iroN* mutant strain, at two different circadian times (10:00 AM, ZT4; 10:00 PM, ZT16). Mice were euthanized at the time point indicated (48–78 h p.i.). For enumerating bacterial cfu, homogenates of colon content, Peyer's patches, mesenteric lymph nodes, and spleen were plated on agar plates containing the appropriate antibiotics [LB plus carbenicillin (Carb), LB plus naladixic acid (Nal)]. Competitive index was calculated by dividing the output ratio (cfu of WT/cfu of *iroN* mutant) by the input ratio (cfu of WT/cfu of *iroN* mutant). Mice used as control were housed in the same conditions as infected mice, pretreated with streptomycin, and left uninfected. Additional information can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank all members of the P.S.-C. and M.R. laboratories for help and discussions; and G. Servillo, M. A. Della Fazia, L. Romani, S.-P. Nuccio, and K. Eckel-Mahan for reagents, discussion, and critical reading of the manuscript. Work in the P.S-C. laboratory was supported by National Institutes of Health (NIH) Grant R01-GM081634 and Sirtris Pharmaceuticals, Inc.; work in the M.R. laboratory was supported by Public Health Service Grants AI083619 and AI083663; and work in the P.B. laboratory was supported by NIH Grants LM010235-01A1 and 5T15LM007743 and National Science Foundation Grant MRI EIA-0321390. J.Z.L. was supported by NIH Immunology Research Training Grant T32 AI60573 and by an American Heart Association Predoctoral Fellowship.

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