Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1

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The response to an innate immune challenge is conditioned by the time of day, but the molecular basis for this remains unclear. In myeloid cells, there is a temporal regulation to induction by lipopolysaccharide (LPS) of the proinflammatory microRNA miR-155 that correlates inversely with levels of Bmal1. Bmal1 in the myeloid lineage inhibits activation of NF-κB and miR-155 induction and protects mice from LPS-induced sepsis. Bmal1 has two miR-155–binding sites in its 3’-UTR, and, in response to LPS, miR-155 binds to these two target sites, leading to suppression of Bmal1 mRNA and protein in mice and humans. miR-155 deletion perturbs circadian function, gives rise to a shorter circadian day, and ablates mRNA and protein in mice and humans. miR-155 deletion perturbs circadian function, gives rise to a shorter circadian day, and ablates mRNA and protein in mice and humans. miR-155 deletion perturbs circadian function, gives rise to a shorter circadian day, and ablates mRNA and protein in mice and humans.

****C****ellular molecular clocks entrain the body to deal with periodic daily changes in the environment. They anticipate and coordinate physiological, behavioral, and biochemical responses. Clocks influence a myriad of fundamental processes such as activity, feeding behavior, body temperature, cell cycle regulation, and hormonal secretion and, as such, are central to the coordination of highly integrated response systems such as metabolism, cardiovascular homeostasis, and immune function (1).

The type and magnitude of the immune response to an inflammatory challenge alters significantly throughout the day, probably to respond optimally at times when the host is most likely to encounter pathogens or danger signals and to optimize the opportunity for resolution of inflammation and recovery (2). In mice, death from sepsis is greatest when the animals begin to transition into the active phase (3–5), and this correlates with an increase in immune cell number (6), immune cell trafficking (4, 7), and circadian gated cytokine production from immune cells (6, 8). Chronic disruption of the molecular clock in mice via jet lag leads to enhanced LPS-induced sepsis, and peritoneal macrophages harvested from these mice produce a greater amount of IL6 in response to LPS (9).

However, the molecular basis underlying the circadian control of innate immunity is still not fully understood. Evidence exists for circadian oscillations of some Toll-like receptors (TLRs) and some of their downstream effector genes (6, 10). TLR9, a receptor for CpG-rich DNA, is controlled by Bmal1 and CLOCK promoter binding (11). Bmal1 and CLOCK are basic helix-loop–helix PAS (bHLH/PAS) transcription factors that drive oscillatory gene expression and lie at the core of molecular clockworks. Bmal1 has also been shown to attenuate NF-κB activity by sequestering CLOCK. CLOCK is required for acetylation of p65, a key event for NF-κB transactivation (12) and downstream cytokine production. Rhythmic oscillation in the numbers of Ly6C\(^{hi}\) monocytes in circulation and the magnitude of recruitment of these cells into inflamed tissue is dependent on Bmal1 (4). As cells lacking Bmal1 lose circadian expression of many clock components, the effect of Bmal1 on inflammation may be due to other clock components acting as intermediaries. For example, the expression of the clock component Rev-Erb\(\alpha\), a nuclear receptor that functions as a transcriptional repressor, is regulated positively by Bmal1 (13, 14). REV-ERB\(\alpha\) has been shown to act as the rhythmic repressor of proinflammatory cytokine production, in particular via repression of IL6 (8). REV-ERB\(\alpha\) can repress via recruitment of the NCoR–HDAC3 complex (14) but also through inhibition of enhancer-derived RNAs (15).

miRNAs are estimated to control more than 30% of the human protein-coding genome (16). Evidence that miRNAs can regulate and be regulated by aspects of the molecular clock exists in Drosophila (17) and mice (18). Cheng et al. (19) identified miR-219 and miR-132 as regulated, respectively, by the molecular clock and light imposing on the suprachiasmatic nucleus (which contains the master clock). miR-219 regulates the length of the circadian day and miR-132 modulates the phase-shifting capacity of light. REV-ERB\(\alpha\) regulates miR-122 in the liver (20).

**Significance**

The circadian clock allows an organism to anticipate daily changes imposed by the environment. The response to LPS is altered depending on time of day; however, the molecular mechanisms underlying this are unclear. We find that the clock in myeloid cells plays a role in LPS-induced sepsis by altering NF-κB activity and the induction of the microRNA miR-155. LPS causes the repression of Bmal1 via the targeting of miR-155 to two seed sequences in the 3’‐untranslated region of Bmal1. Lack of miR-155 has profound effects on circadian function and circadian induction of cytokines by LPS. Thus, the molecular clock is using miR-155 as an important regulatory component to control inflammation variably across the circadian day in myeloid cells.


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and miR-142-3p is controlled by the BMAL1:CLOCK heterodimer and, in turn, can target Bmal1 (21).

Here, we provide evidence that miR-155, a proinflammatory microRNA induced by TLRs (22, 23), controls Bmal1 miRNA and protein levels in myeloid cells, leading to alterations in clock function and circadian control of inflammation. In agreement with a previous study (4), we demonstrate that the time-dependent variation in the consequences of acute sepsis is reliant on the level of myeloid BMAL1. The molecular clock attenuates inflammation via its effects on NF-kB (12) and can suppress proinflammatory cytokines and miR-155. MiR-155 via its effects on BMAL1 can alter circadian function, including the control of inflammatory cytokines. We identify miR-155 as a critical posttranscriptional repressor of Bmal1, providing a direct link between the molecular clock, a microRNA, and immune function in macrophages.

**Results**

**Mice Deficient in Myeloid BMAL1 Have an Increased Risk of LPS-Induced Sepsis with Heightened Induction of the Proinflammatory microRNA miR-155 and NF-κB Activity.** We and others (4, 8) have used the Bmal1−/− Lys-MCre mouse—in which BMAL1 is removed from the myeloid lineage (Fig. S1 A and C) and which causes suppression of the clock components Rev-Erba and Per2 (Fig. S1C)—to study further the effect of the molecular clock on LPS challenge. As reported (8), these mice retain normal acicular clock, a microRNA, and immune function in macrophages.

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![Fig. 1. The magnitude of the circadian response to sepsis and inflammation correlates with induction of the microRNA miR-155.](https://www.pnas.org/cgi/doi/10.1073/pnas.1501327112 Curtis et al.)
from peritoneal cells lacking BMAL1. Peritoneal macrophages harvested at ZT0 and ZT12 and challenged with LPS resulted in a reduction in Bmal1 expression at both time points (Fig. 2A). Similarly, BMDMs treated with LPS had a significant reduction in Bmal1 mRNA (Fig. 2B) and protein (Fig. 2C) over time, correlating with an increase in miR-155 (Fig. S2J). Among a range of TLR agonists used at a single concentration, LPS caused significant repression of Bmal1 (Fig. 2D). Infection of the macrophage cell line, RAW264.7, with the pathogens Salmonella typhimurium and Escherichia coli also caused a significant up-regulation of miR-155 (Fig. 3A). Use of the software TargetScan, two predicted miR-155 microRNA miR-155 Binding to Two Sites Within the 3′ UTR of Bmal1 in Macrophages was first confirmed by in vitro luciferase assays (Fig. 3B). Using the software TargetScan, two predicted miR-155 binding sites (GCATTAA) were identified at positions 40 and 235 within the 3′ UTR of Bmal1 mRNA (Fig. 3B). Two short interfering RNA (siRNA) oligonucleotides were designed to target the miR-155 binding sites (Fig. S4B). Transfection of the siRNA oligonucleotides into BMDMs along with the antagomir to miR-155 followed by LPS resulted in a reduction in luciferase reporter activity in the absence of miR-155 (Fig. 3C). The magnitude of repression of BMAL1 protein was attenuated in the absence of miR-155 (Fig. 3G and H and Fig. S4A). Consistent with the effect of the miR-155 antagonist on TNFa (Fig. 3E), lack of miR-155 also suppressed the induction of Tnfa mRNA from BMDMs (Fig. 3F). Morpholinos against each of the miR-155 target sites within the Bmal1 3′ UTR [site 1 at position 40 and site 2 at position 235 (Fig. S4B)] were transfected for 24 h into BMDMs along with the antagonist to miR-155 followed by LPS treatment for 24 h. When analyzed for Bmal1 expression, the antagonist and the morpholinos blocked the ability of LPS to repress Bmal1 protein (Fig. 3F). The induction of mature miR-155 by LPS was not significantly affected by transfection of the morpholinos (Fig. 3K). We also cloned the Bmal1 3′ UTR into a luciferase plasmid and mutated both miR-155–binding sites. With the wild-type plasmid both LPS and miR-155 overexpression caused a reduction in luciferase (Fig. 3L, black isobars). When both sites were mutated, no reduction in luciferase was observed with either MiR-155 overexpression or LPS stimulation (Fig. 3L, striped isobars).

**Down-Regulation of Bmal1 upon LPS Challenge Is Coincident with miR-155 Induction in Mice.** Peritoneal macrophages harvested at ZT0 and ZT12 and challenged with LPS resulted in a reduction in Bmal1 expression at both time points (Fig. 2A). Similarly, BMDMs treated with LPS had a significant reduction in Bmal1 mRNA (Fig. 2B) and protein (Fig. 2C) over time, correlating with an increase in miR-155 (Fig. S2J). Among a range of TLR agonists used at a single concentration, LPS caused significant repression of Bmal1 (Fig. 2D). Infection of the macrophage cell line, RAW264.7, with the pathogens Salmonella typhimurium and Escherichia coli also caused a significant up-regulation of miR-155 (Fig. 3A). Use of the software TargetScan, two predicted miR-155 microRNA miR-155 Binding to Two Sites Within the 3′ UTR of Bmal1 in Macrophages was first confirmed by in vitro luciferase assays (Fig. 3B). Using the software TargetScan, two predicted miR-155 binding sites (GCATTAA) were identified at positions 40 and 235 within the 3′ UTR of Bmal1 mRNA (Fig. 3B). Two short interfering RNA (siRNA) oligonucleotides were designed to target the miR-155 binding sites (Fig. S4B). Transfection of the siRNA oligonucleotides into BMDMs along with the antagomir to miR-155 followed by LPS resulted in a reduction in luciferase reporter activity in the absence of miR-155 (Fig. 3C). The magnitude of repression of BMAL1 protein was attenuated in the absence of miR-155 (Fig. 3G and H and Fig. S4A). Consistent with the effect of the miR-155 antagonist on TNFa (Fig. 3E), lack of miR-155 also suppressed the induction of Tnfa mRNA from BMDMs (Fig. 3F). Morpholinos against each of the miR-155 target sites within the Bmal1 3′ UTR [site 1 at position 40 and site 2 at position 235 (Fig. S4B)] were transfected for 24 h into BMDMs along with the antagonist to miR-155 followed by LPS treatment for 24 h. When analyzed for Bmal1 expression, the antagonist and the morpholinos blocked the ability of LPS to repress Bmal1 protein (Fig. 3F). The induction of mature miR-155 by LPS was not significantly affected by transfection of the morpholinos (Fig. 3K). We also cloned the Bmal1 3′ UTR into a luciferase plasmid and mutated both miR-155–binding sites. With the wild-type plasmid both LPS and miR-155 overexpression caused a reduction in luciferase (Fig. 3L, black isobars). When both sites were mutated, no reduction in luciferase was observed with either MiR-155 overexpression or LPS stimulation (Fig. 3L, striped isobars).

**LPS-induced miR-155 Targets Bmal1 in Human Macrophages and Adipose Tissue.** In human macrophages treated with LPS, hBmal1 mRNA (Fig. 4A) and protein (Fig. 4B, compare lane 7 with lane 8) were reduced at 4 and 24 h, respectively, along with a progressive increase in hmiR-155 over time (Fig. 4C). This reduction in human BMAL1 with LPS was a direct effect of miR-155 as levels of BMAL1 protein in human peripheral blood mononuclear cells (PBMCs) transfected with an antagonist to hmiR-155 were not suppressed by LPS [Fig. 4D; compare lanes 1–3 (antagonist) to 4–6 (scrubbed)]. The hmiR-155 antagonist was also capable of suppressing the induction of phosphorylated p65 in PBMCs [Fig. 4E; compare lanes 2–4 (scrubbed) with 8–10 (antagonist)]. Subcutaneous adipose tissue derived from 14 healthy volunteers was collected before and after an 1 h bolus of LPS (3 ng/kg) (Table 1). Gene expression analysis by microarray established that MIR-155HG was significantly increased on microarray after 4 h of exposure to LPS together with a significant reduction in Bmal1 (Table 1). An increase in PER2 and a decrease in REV-ERBα was observed in these human biopsies upon LPS activation with no change in CLOCK or RORα. Remarkably, the same effects on those genes were observed in mouse peritoneal cells harvested at ZT0 and treated with LPS for 4 h (Fig. S3D–H). As REV-ERBα was the only other clock gene analyzed that was repressed by LPS in mouse peritoneal cells at ZT0 and treated with LPS for 4 h (Fig. S3D–H), this suggests that REV-ERBα expression is regulated by LPS in peritoneal cells and that REV-ERBα deletion, and LPS did not cause significant repression in comparison with
**Discussion**

Previous reports of a rhythm in the susceptibility of mice to LPS, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *S. typhimurium* challenge (3–5, 26) suggest that the molecular clock may play a fundamental role in controlling the mammalian immune response. Susceptibility to lethality is greatest when the challenge occurs close to the transition into the activity phase. Gibbs et al. demonstrated that this period of susceptibility correlates with a heightened production of proinflammatory cytokines with activation of REV-ERBα, a BMAL1 target, attenuating inflammation, in particular via repression of IL6 (8). A more recent study reported that *Bmal1* in the myeloid lineage controls the number of circulating Ly6Cγ monocytes (4). When *Bmal1* was absent from these cells, their recruitment to the site of infection was amplified as BMAL1 directly regulates *Cd2* transcription. However, identifying the mechanisms that intersect the immune and clock systems have been challenging.

Here we show that the protection from LPS-induced lethality at ZT0 in comparison with ZT12 correlates with reduced induction of the proinflammatory microRNA miR-155, but increased induction of the anti-inflammatory cytokine IL10. The absence of BMAL1 in the myeloid lineage sensitizes the mice to LPS along with a corresponding increase in miR-155, but a decreased production of IL10. The heightened proinflammatory milieu of myeloid cells may in part be responsible for the enhanced lethality observed in *Bmal1*-deficient mice. However, as has been reported, uncontrolled trafficking of monocytes (4) and possibly other myeloid populations such as neutrophils, also known to oscillate (7), may impact on the phenotype in *Bmal1*-deficient mice.

A number of mechanisms may explain the circadian control on miR-155 induction by LPS. First, the promoter of the *MIr-155HG* free-running period than the controls (t of 25.42 ± 0.11 h in WT compared with 24.83 ± 0.17 h in miR-155−/− animals; P = 0.013; Fig. 5C and Fig. S5).

Consistent with previous results, peritoneal cells from miR-155−/− mice harvested at ZT0 and ZT12 and treated with LPS for up to 24 h were unable to repress *Bmal1* in comparison with wild-type cells (Fig. S5D). Strikingly, the circadian effect on TNFα production (i.e., higher induction from ZT12-treated cells versus ZT0-treated cells) was lost from cells lacking miR-155, with similar production of TNFα by LPS at both ZTs (Fig. 5E).

**Altered Circadian Function and Clock-Controlled Cytokine Production in Mice Lacking miR-155.** Peritoneal cells were extracted at ZT0 and ZT12 to assay circadian expression under steady-state conditions. miR-155 acts as a repressor of *Bmal1* even under steady-state conditions as its deletion caused higher basal levels of *Bmal1* at ZT0 (Fig. 5A). Given this observation, we assayed a free-running period in mice lacking miR-155. Over the course of a 4-wk-free run in constant darkness (DD), the free-running period was shortened in miR-155−/− mice compared with WT (t of 23.81 ± 0.02 h in WT compared with 23.66 ± 0.04 h in miR-155−/− animals; P = 0.013; Fig. 5B and Fig. S5). To examine whether this difference in τ would persist in constant light, animals were then transferred into constant light (LL) for 4 wk. Again, the miR-155−/− animals displayed a significantly shorter control (Fig. S3F). Similarly, Rev-Erbα expression was not reduced with LPS in miR-155−/− BMDMs (Fig. S3J).

**Fig. 3.** *Bmal1* is repressed by the microRNA MiR-155 under basal and LPS conditions. (A) Schematic of *Bmal1* 3′ UTR illustrating position of the two miR-155-binding sites identified with the software TargetScan. iBMDMs transfected with a miR-155 mimic and analyzed for expression of (B) *Bmal1* mRNA and protein (n = 3). NC, negative control for mimic; LF, Lipofectamine; MiR-155OE, overexpression of MiR-155 mimic. iBMDMs transfected with either a negative control for antagonim (NC) or an antagonim (Ant.) miR-155, treated with LPS (100 ng/mL), and analyzed for expression of (D) *Bmal1* and (E) TNFα levels by ELISA (n = 3). WT or miR-155−/− BMDMs treated with LPS (100 ng/mL) for the indicated time and analyzed for expression of (F) *Bmal1* and (G) protein levels by immunoblot. (H) Densitometry values of immunoblots from G and Fig. S4 (n = 3–4), and (I) TNFα mRNA (n = 3). BMDMs transfected with a scrambled control morpholino (Scr), MiR-155 antagonim (Ant.) morpholino against the MiR-155 site in *Bmal1* at position 40 (site 1), and morpholino against the MiR-155 site in *Bmal1* at position 235 (site 2) were treated with LPS (100 ng/mL) for 24 h and analyzed for expression of (J) *Bmal1* and (K) *Bmal1*′ 3′ UTR construct with LPS induction (100 ng/mL) or overexpression of MiR-155 mimic with wild-type luciferase construct or double (Mut. Site 1 + 2) mutations of miR-155-binding sites (n = 3). C; control; NC, negative control for mimic; 155, overexpression of miR-155 mimic. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

**Fig. 4.** MiR-155 targets BMAL1 in humans and suppresses NF-κB activity. Human macrophages from four donors stimulated ex vivo with LPS (100 ng/mL) for the indicated times and analyzed for expression of (A) BMAL1 mRNA, (B) BMAL1 protein, and (C) miR-155. (D) hPBMCs were transfected with an antagonist to miR-155 or scrambled control and then treated with LPS for indicated times and immunoblotted for BMAL1 and β-actin as control. Values provided are relative band intensity of BMAL1/β-actin. (E) hPBMCs were transfected with an antagonist to miR-155 or scrambled control and then treated with LPS for indicated times and immunoblotted for phosphorlated p65 at Serine S32 and total p65 as control. Values provided are relative band intensity of phospho-p65/total p65. *P ≤ 0.05.
contains an NF-κB site (25, 27), and NF-κB inhibitors attenuate the induction of MiR-155HG (28). Splenger et al. revealed that in fibroblasts CLOCK acetates and thus activates p65 via increased levels of p65 phosphorylation and that BMAL1 heterodimerization with CLOCK suppressed this activity (12). We also confirm the effect of BMAL1 on NF-κB activity in peritoneal cells, so BMAL1 could regulate miR-155 and some proinflammatory cytokines including TNFα (29) via its effects on p65. The transcription factor ETS2 is responsible for miR-155 induction in LPS-activated macrophages, and IL10, via inhibition of ETS2, can suppress miR-155 induction (25). In addition to our observations that myeloid cells lacking BMAL1 produce less IL10, we observe also that IL10**−** BMMDMs have high levels of miR-155 and low levels of Bmal1 under steady-state conditions. Therefore, BMAL1, through its regulation of IL10, could inhibit miR-155. This could be controlled either directly by BMAL1 or via another clock component such as Rev-Erbα. Altogether, our results confirm that BMAL1 within myeloid cells can attenuate production of miR-155 and proinflammatory cytokines in response to LPS, an effect that might impact on leukocyte activation and trafficking during sepsis occurrence.

However, it is important to note that, although BMAL1 can regulate the p65 subunit of NF-κB, not all LPS-induced NF-κB-regulated genes have a circadian gating (S). This could be because there are multiple clock-regulated components that code responses in a cell-specific manner (including REV-ERBα, which will repress certain genes) and because p65 is controlled by several other proteins other than CLOCK. It is also possible that there is circadian regulation of chromatin architecture, which limits accessibility for NF-κB. These aspects require further analysis.

Given the importance of BMAL1 in modulating the inflammatory response, we next considered the effect of LPS on Bmal1. We hypothesized that for LPS to elicit its effect on the inflammatory process it would attenuate BMAL1. LPS and bacterial infections significantly decreased the levels of Bmal1 mRNA and protein, an event coincident with increased levels of miR-155. miR-155 was a strong candidate as a regulator of Bmal1 ab initio, given that it is up-regulated rapidly upon TLR activation (30) and that its induction is dependent on the level of BMAL1 in myeloid cells. Indeed, suppression of Bmal1 mRNA and protein upon LPS activation is lost or attenuated in macrophages lacking miR-155, which also suppresses induction of Tnfα transcript. This was confirmed further with an antagonist to miR-155, which protects against suppression of Bmal1 by LPS while also attenuating Tnfα induction. Some repression of BMAL1 protein to LPS was still detectable in the absence of miR-155. This suggests that, in addition to the posttranscriptional control that miR-155 exerts on Bmal1, there may exist posttranslational modifiers acting at the protein level. Morpholinos generated against the two miR-155-binding sites in the Bmal1 3′ UTR each inhibit the ability of LPS to repress Bmal1 in BMDMs, confirming further the direct effect of miR-155 on Bmal1. Therefore, controlled removal of macrophage Bmal1 by miR-155 is required to mount an acute inflammatory response. We found that LPS repressed human BMAL1 in macrophages with a corresponding increase in hMiR-155 when treated in vitro. The human BMAL1 3′ UTR has only one miR-155-binding site in comparison with the mouse sequence that has two. However, we could completely block the repression of human BMAL1 by LPS using an antagonist to human miR-155. We could also suppress the level of p65 phosphorylation using the antagonist to human miR-155. This confirms further that modulation of the Bmal1/miR-155 axis can affect the downstream activity of NF-κB and the inflammatory process. An increase in the hMiR-155HG and a decrease in hBmal1 was also evident in the adipocyte biopsies obtained from patients treated with low-dose LPS. Indeed, there is remarkable translational concordance between the effects on Clock, Period2, Rev-Erbα, and RoRa observed in human adipocytes with those seen on mouse peritoneal cells treated with LPS. Rev-Erbα was the only other clock gene analyzed that was repressed by LPS. The repression of Rev-Erbα by LPS was in part protected with loss of miR-155, whereby BMAL1 would remain high. Therefore, some of the proinflammatory effects featured with loss of BMAL1, such as increased IL6, may be due to loss of REV-ERBα.

Under steady-state conditions, miR-155 also negatively regulates Bmal1 expression at ZT0. This led us to investigate whether miR-155 would have effects on the central clock. It is intriguing that miR-155**−** mice display a shorter period length. Although this observation requires further investigation, it is consistent with the role of miR-155 as a transcriptional repressor of Bmal1, as mice lacking Rev-Erbα, a potent transcriptional repressor of Bmal1, have

**Table 1. Clock gene expression from human endotoxemia study**

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<th>Gene</th>
<th>Pre-LPS (mean)†</th>
<th>SD</th>
<th>Post-LPS (mean)†</th>
<th>SD</th>
<th>P value‡</th>
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<td>BMAL1</td>
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<td>0.0006</td>
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**P ≤ 0.01 and ***P ≤ 0.001.
† Paired t tests were performed on pre-LPS and 4-h post-LPS expression values to determine significance.
‡ A paired t test was performed on pre-LPS and 4-h post-LPS expression values to determine significance.

**Fig. 5.** Altered clock function and clock-gated cytokine responses in mice lacking MiR-155. (A) Peritoneal cells harvested from WT and miR-155**−** mice at ZT0 or ZT12 were immediately lysed and analyzed for Bmal1 (n = 3–4). Period length of control and miR-155-deficient mice in (B) constant dark conditions (DD) and (C) constant light conditions (LL) (n = 9–14). (D) Peritoneal macrophages harvested from WT and miR-155 mice at ZT0 and at ZT12 and treated immediately ex vivo with LPS (100 ng/mL) for 4, 8, and 24 h and analyzed for expression of Bmal1 by area under a curve (AUC). (E) Peritoneal macrophages were harvested from WT and miR-155**−** mice at ZT0 and at ZT12 and treated immediately ex vivo with LPS (100 ng/mL) for 24 h, and supernatants were analyzed by ELISA for TNFα (n = 4). (F) Schematic model to depict the circadian effect on LPS activation via the circadian control of miR-155 on Bmal1 in myeloid cells. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.
a similar, albeit more pronounced, effect on activity (13). In addition, lack of miR-155 in myeloid cells ablates the circadian effect on evoked TNFα normally observed between ZT0 and ZT12.

Collectively, these studies identify miR-155 as an important regulatory component of circadian function and provide a description of a previously unidentified mechanism by which the circadian clock controls the innate immune response. miR-155 is believed to potentially function in macrophages, in part through its effects on the TLR4 repressors Ship1 and SocS3 (31, 32) and its ability to stabilize Tnfα (28). miR-155 is controlled by the molecular clock, leading to its circadian induction. We show also that high levels of miR-155 and the consequent targeting of BMAL1 might lead to a proinflammatory state through activation of the NF-κB complex. To our knowledge, this is the first report of a miRNA, integral to the immune system, affecting the temporal and inflammatory variability of the molecular clock. Furthermore, BMAL1 or its targets such as REV-ERBs negatively regulate innate immunity such that LPS must repress BMAL1 itself via miR-155 should BMAL1 be present at a particular time of day. Innate immunity therefore uses the control of BMAL1 by miR-155 to control the circadian inflammatory response in myeloid cells. Our findings provide insight into the temporal control of inflammation, which could have consequences for our understanding of the pathogenesis of inflammation and infectious diseases where circadian regulation is known to be important.

Materials and Methods

LPS Survival in Mice. Mice were maintained in a controlled environment for 1 wk before the LPS study. Mice were injected either at ZT0 or ZT12 with LPS derived from E. coli serotype 055:B5 (Siga Aldrich) in sterile PBS at 25 mg/kg by the i.p. route and monitored for 6–8 d. All animal studies were performed in accordance with the guidelines approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania and the Animal Research Ethics Committee at Trinity College Dublin.

Peritoneal Exudate Cells. Mice were euthanized at indicated times and cells were collected by peritoneal lavage. Cells were seeded at 1 x 10⁶ cells per well in serum-free media, and after 45 min nonadherent cells were washed out and attached cells were harvested immediately for RNA and protein analysis, or LPS was added to the attached cells for specified times and then cells were harvested for RNA, and protein and supernatants were harvested for ELISAs.

Transfections. Wild-type iBMDMs, a gift from Douglas Golenbock, University of Massachusetts Medical School, Worcester, MA, were seeded at 1 x 10⁶ cells/well. On the next day, cells were transfected with 1 μM of miR-155 premiR or the scrambled oligonucleotide premiR negative control (Ambion) using Lipofectamine 2000 (Invitrogen) for 8 h. Media were replaced with fresh antibiotic-free media, and cells were harvested 24 h later. For the antagonist studies in iBMDMs and RPE-1, 50 nM of the miR-155 antagonist or negative control (Ambion) was used. For BMDCs, morpholinos against the two miR-155-binding sites (1 μM) were transfected with the reagent Endo-Porter (33). Twenty-four hours later, cells were treated to LPS (100 ng/ml) and harvested. For luciferase reporter studies, the complete 3’ UTR of Bmal1 containing the two predicted miR-155-binding sites or with the sites mutated was inserted into the dual luciferase Psichek2 reporter vector (Promega). iBMDMs were plated in a six-well format and cotransfected using Lipofectamine 2000, and luciferase activity was measured after 48 h.

Human Subjects Study Protocol. The institutional review board of the University of Pennsylvania approved the protocol and all subjects gave written informed consent.

Data Analysis. Results are presented as mean ± SEM. Statistical analysis was performed using Prism 5. Differences were compared by using analysis of variance followed by Student–Newman–Keuls post hoc analysis and/or paired or unpaired Student’s t test, as appropriate. Significance values are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. For survival data, a log-rank (Mantel–Cox) test was used.

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