The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines

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Diurnal variation in inflammatory and immune function is evident in the physiology and pathology of humans and animals, but molecular mechanisms and mediating cell types that provide this gating remain unknown. By screening cytokine responses in mice to endotoxin challenge at different times of day, we reveal that the magnitude of response exhibited pronounced temporal dependence, yet only within a subset of proinflammatory cytokines. Disruption of the circadian clockwork in macrophages (primary effector cells of the innate immune system) by conditional targeting of a key clock gene (*bmal1*) removed all temporal gating of endotoxin-induced cytokine response in cultured cells and in vivo. Loss of circadian gating was coincident with suppressed *rev-erbα* expression, implicating this nuclear receptor as a potential link between the clock and inflammatory pathways. This finding was confirmed in vivo and in vitro through genetic and pharmacological modulation of *REV-ERBα* activity. Circadian gating of endotoxin response was lost in *rev-erbα*$^{-/-}$ mice and in cultured macrophages from these animals, despite maintenance of circadian rhythmicity within these cells. Using human macrophages, which show circadian clock gene oscillations and rhythmic endotoxin responses, we demonstrate that administration of a synthetic REV-ERB ligand, or genetic knockdown of *rev-erbα* expression, is effective at modulating the production and release of the proinflammatory cytokine IL-6. This work demonstrates that the macrophage clockwork provides temporal gating of systemic responses to endotoxin, and identifies REV-ERBα as the key link between the clock and immune function. REV-ERBα may therefore represent a unique therapeutic target in human inflammatory disease.

Circadian clocks provide organisms with an internal mechanism to maintain temporal order in a rhythmic environment. The molecular clockwork is highly conserved in man and animals, and orchestrates the daily patterning of diverse physiological processes such as sleep/wake cycles, feeding, and metabolism. Many diseases exhibit circadian rhythmicity in their pathology, and lifestyles that disrupt inherent timing systems, such as chronic shift work, are associated with an increased risk of cancer, metabolic disorders, and cardiovascular and cerebrovascular disease (1). Inflammatory diseases in particular exhibit strong time-of-day symptoms. For example, rheumatoid arthritis (RA) has a strong diurnal variation in disease expression, which is accompanied by fluctuations in circulating IL-6 concentration (2). In mice, significant temporal dependence of LPS-induced endotoxic shock has been reported (3), and circadian disruption mimicking jet lag can greatly magnify LPS response (4). Many facets of immune function show diurnal variation, and recent studies have revealed that macrophages, important regulators of innate immune responses, exhibit robust circadian oscillations in gene expression, including genes responsible for pathogen recognition and cytokine secretion (5, 6). However, the molecular mechanism, which couples immune function to the circadian clockwork, remains unknown.

In mammals, circadian rhythms are driven by a complex of feedback loops centered on the transcriptional activators CLOCK and BMAL1, and transcriptional repressors PERIOD (PER) and CRYPTOCHROME (CRY). These feedback loops generate a repetitive transcriptional/translational oscillator with a period of ~24 h. A stabilizing loop within the clockwork is provided by CLOCK/BMAL1 transactivation of the nuclear receptors RORα and REV-ERBα, which feedback to activate or repress BMAL1 transcription (respectively) by competing for shared RORE promoter elements. In addition, REV-ERBα has been implicated in numerous physiological processes outside the clock, typically mediated through recruitment of nuclear receptor corepressor-1 (NCoR), histone deacetylase 3 (HDAC3), and subsequent target gene repression (7).

In the current study, we used circadian variation in endotoxin response to define key components involved in clock gating of the innate immune response. Using IL-6 as a primary biomarker, we show that temporal dependence of murine responses to endotoxin challenge is abolished when the clock is disrupted in macrophages. *Rev-erbα*$^{-/-}$ mice exhibit a similar loss of temporal gating, even though normal circadian function is retained in macrophages. Using pharmacological and genetic targeting in human macrophage cells, we further demonstrate REV-ERB control over a selective set of genes involved in human innate immunity, including *il6*. These data demonstrate that temporal gating of proinflammatory cytokine responses are mediated by the macrophage clock, from which REV-ERBα acts as a critical intermediary between the core clockwork and inflammatory pathways.

Results

Cytokine Responses to LPS Are Selectively Gated by the Circadian Clock in Mice. We first used an unbiased multiplex cytokine assay to screen for circadian effects on innate immune responses in mice. Animals were treated with i.p. LPS endotoxin at 0 h circadian time (CT0; start of the rest phase) or CT12 (start of the active phase) and serum collected 4 h later. Analysis revealed that of 22 cytokines measured, 13 were induced by LPS, of which only 5 [IL-6, IL-12(p40), CXCL1, CCL5, and CCL2] showed significant circadian-dependent variation in the magnitude of response (Fig. 1*A* and Table S1). Cytokine concentrations in

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vehicle-treated animals showed no significant time-of-day differences, and TNF-α, which has previously been reported to be rhythmic in cultured macrophages (6), showed no significant time-of-day differences in vivo (Fig. 1*A*). In separate experiments, peritoneal exudate cells (PECs), which are predominantly macrophages based on surface expression of F4/80 and CD11b (Fig. S1*A*), were collected from mice 30 min after LPS administration (at either CT0 or CT12), and cytokine transcript levels measured. In line with circulating protein assays, *il6*, *il12(p40)*, *cxcl1*, and *ccl2* mRNA were rapidly induced by LPS (Fig. S1*B*), and the magnitude of this response was significantly greater at CT12 (Fig. 1*B*). *ccl5* was also significantly induced by LPS, although this was not evident until 120 min postadministration (Fig. S1*B*).

Macrophage Clock Provides Temporal Gating of Cytokine Responses. Macrophages are key responding cells in the innate immune response to LPS, and a prominent source of proinflammatory cytokines. We therefore hypothesized that these cells may orchestrate the temporal variation in endotoxin response. To test this, we generated macrophage-specific *bmal1*$^{-/-}$ mice (*LysM-bmal*$^{-/-}$), which carried a luciferase reporter for the circadian clockwork (mPER2:Luc) (8). *LysM-bmal*$^{-/-}$ mice exhibited normal circadian patterns in wheel-running activity, and retained normal rhythmic activity of the central suprachiasmatic nucleus (SCN) clock (Fig. S2 *A* and *B*). PECs isolated from control WT mice exhibited robust circadian oscillations (Fig. 2*A*). In contrast, *LysM-bmal*$^{-/-}$ mouse-derived cells lacked detectable BMAL1 protein (Fig. 2*B*) and showed no PER2::luc bioluminescence oscillation (although PER2 did retain its acute monophasic response to glucocorticoid; Fig. 2*A*). *Bmal1* deletion caused constitutive nonrhythmic expression of *per2*, *cry1*, and *dbp* and suppressed transcripts for *rev-erbα* and *rev-erbβ* (Fig. 2*C*) in contrast to WT mice, which exhibited pronounced circadian rhythms of these clock genes (Table S2). An arrhythmic *bmal* transcript (exons 5–7) was detectable in PECs from *LysM-bmal*$^{-/-}$ mice (which lack exon 8 of the *bmal* gene). RT-PCR of exon 8 confirmed efficient recombination in *LysM-bmal*$^{-/-}$ PECs (Fig. S2*C*). Although transcription of *rev-erbα* was dramatically repressed in the absence of *bmal1*, a residual rhythm was detected, possibly due to involvement of additional *rev-erbα* regulators, such as BMAL2 (9). To confirm that targeted cells were arrhythmic in vivo, PECs harvested from naïve *LysM-bmal*$^{-/-}$ mice and WT controls at CT0 and CT12 were compared. In WT cells, high-amplitude time-of-day differences were observed for *per2*, *bmal1*, *rev-erbα*, *rev-erbβ*, and *dbp* mRNA, in contrast, *bmal*$^{-/-}$ PECs revealed no significant time-of-day differences in expression (Fig. S2*D*). Thus, using a macrophage-specific targeting strategy, we show that PECs are rendered totally circadian-arrhythmic, both in vivo and in vitro. The functional consequences of macrophage arrhythmicity were tested in vivo and in vitro. In WTs, LPS-induced serum IL-6, CCL5, and IL-12(p40) concentrations were significantly higher after CT12 challenge compared with CT0. A time-dependent effect on the magnitude of the LPS response was not observed in *LysM-bmal*$^{-/-}$ mice, indicating loss of the circadian gating mechanism (Fig. 2*D* and Fig. S3 *A* and *B*). Similarly, though a robust gating of IL-6 release was observed in cultured WT PECs, *LysM-bmal*$^{-/-}$ PECs exhibited no significant gating of IL-6 response when tested at CT0 and CT12 (Fig. 2*E*). These findings demonstrate that the macrophage clock has a profound modulating effect, both on responses of targeted cells cultured in vitro, and on systemic innate immune activation in vivo.

REV-ERBα Links the Macrophage Clock to Inflammatory Processes and Modulates Proinflammatory Cytokine Response. PECs exhibited a profound temporal variation in *rev-erbα* (20-fold difference between CT0 and CT12), which was greatly suppressed in PECs lacking BMAL1, implicating a potential role for REV-ERB in the loss of gating in macrophages. In support, *rev-erbα* has been associated with macrophage toll-like receptor (TLR) signaling (10) and IL-6 gene transcription (11). To test the role of *rev-erbα* in the gating response of IL-6 in vivo, we assessed the endotoxin response in *rev-erbα*$^{-/-}$ mice. As predicted, WT littermates exhibited an LPS-evoked IL-6 response, which was significantly higher at CT12 than CT0. This gating was absent in *rev-erbα*$^{-/-}$ mice, with elevated serum responses at CT0 similar to those at CT12 (Fig. 3*A*). To determine whether this was due to abolition of the immune response rhythm, or simply a phase shift undetected by 12-h sampling, the LPS-driven IL-6 response was measured in a further study at 6-h time points across the circadian day (Fig. S3*C*). In contrast to WT mice, at all time points, responses to LPS were similar, and it is evident that rhythmic immune responses are abolished in *rev-erbα*$^{-/-}$ mice. Extension of these studies to in vitro culture of PECs revealed loss of a gated

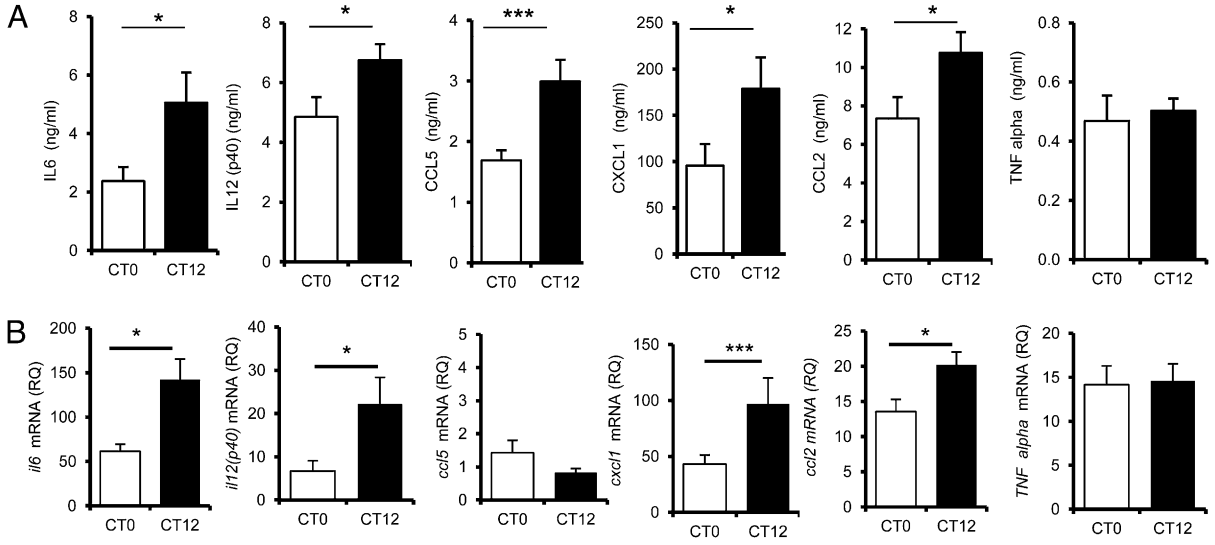

Fig. 1. Circadian gating of murine cytokine responses to LPS. (*A*) Serum cytokines were quantified 4 h after i.p. LPS administration at either CT0 or CT12. IL-6, IL-12(p40), CCL5, CXCL1, and CCL2 (but not TNF-α) showed significantly higher levels after CT12 challenge vs. CT0 (n = 7–9, two-way ANOVA, post hoc Bonferroni). (*B*) mRNA was isolated from PECs harvested 30 min after LPS treatment at either CT0 or CT12. Levels of cytokine mRNA were quantified (relative to β-actin) and are presented in relation to expression levels in PECs harvested at CT0 from vehicle-treated mice (n = 6–7, t test).

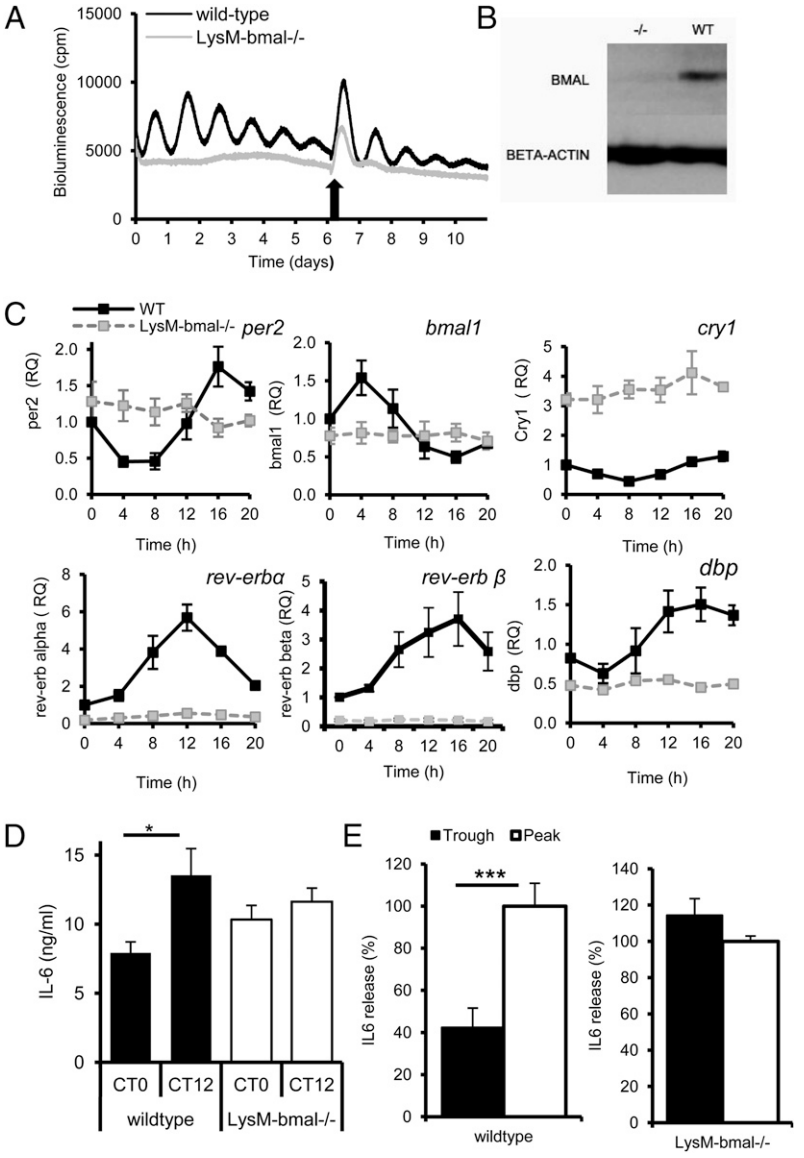

Fig. 2. Generation of *LysM-bmal*$^{-/-}$ mice. (*A*) PECs cultured from *LysM-bmal*$^{-/-}$ on a PER2::luc background are arrhythmic in contrast to WT mice. Arrow indicates application of dexamethasone (representative of four trials). (*B*) Western blotting confirms loss of BMAL protein in targeted macrophages. (*C*) Isolated PECs from *LysM-bmal*$^{-/-}$ mice show altered expression of core clock genes in culture (n = 3); transcripts are reported relative to time 0 in WT mice. (*D*) In vivo LPS challenge at CT0 and CT12 elicits comparable levels of IL-6 release in *LysM-bmal*$^{-/-}$ mice, whereas WT mice retain the gated responses (n = 8–9, one-way ANOVA, Bonferroni t test). (*E*) In vitro stimulation of PECs from *LysM-bmal*$^{-/-}$ mice at opposing time points confirmed loss of gating in these cells (n = 3–4, t test).

IL-6 LPS response in *rev-erbα*$^{-/-}$ cells (Fig. 3*B*). Importantly, this loss of gating was not due to a general disruption of the macrophage clockwork, because PER2::luc bioluminescence recordings from *rev-erbα*$^{-/-}$ PECs confirmed that these cells remained strongly rhythmic (Fig. 3*C*). In addition, quantitative PCR (qPCR) profiling of clock gene expression confirmed that *rev-erbα*$^{-/-}$ PECs retained functional circadian oscillations, with high-amplitude rhythms in *dbp* and *rev-erbβ* (Fig. 3*D*). Intriguingly, *bmal1* also retained rhythmic expression in these cells, implying retention of rhythmic E-box–mediated transactivation in this cell population; this contrasts with an earlier study (12) that demonstrated reduced amplitude of *bmal1* mRNA oscillations in the liver of *rev-erbα*$^{-/-}$ mice throughout the circadian day, an observation we have confirmed in both liver and lung.

REV-ERB Action on Human Macrophage Cells. Our data in mice reveal IL-6 as a major clock-regulated cytokine. In humans, circulating levels of IL-6 are also strongly rhythmic, and IL-6 has been identified as a predictive marker for RA (2). In contrast, IL-8 concentrations are not subject to time-of-day regulation in man. We observed primary human monocyte-derived macrophages (MDMs) to exhibit rhythmic clock gene expression (Fig. S4*A*) and therefore investigated gating responses to LPS stimulation of clock-synchronized MDMs at 4-h intervals over 28 h. Cells were harvested 4 h after treatment. LPS induction of *il6* showed significant variation in transcript response, with strong induction 16 h postsynchronization (Fig. 4*A*). Interestingly, unstimulated MDMs showed low-amplitude circadian variation in baseline levels of *il6* transcription (cosinor analysis: period = 19.6 h, $P < 0.05$), peaking 8–16 h after synchronization (Fig. S4*A*). Our earlier studies reported action of a REV-ERB ligand (GSK4112) on circadian controlled circuits (13). We used GSK4112 to test whether the enhanced REV-ERB repressive activity induced by this ligand could diminish LPS-driven IL-6 release in MDM cells and primary human alveolar macrophages. GSK4112 treatment inhibited IL-6 protein secretion in both cell types, with a greater potency in alveolar macrophages, yet IL-8 induction was not inhibited in either cell type (Fig. 4*B*).

To further explore the mechanisms of REV-ERB control of the cytokine response, we used a human myelomonocytic cell line (THP-1). Consistent with our data on primary macrophages, GSK4112 treatment of THP-1 cells inhibited LPS induction of IL-6, but not IL-8 (Fig. 4*C*). Because REV-ERBα may regulate TLR expression (10), we measured *tlr2* and *tlr4* and found that neither was regulated by the ligand (Fig. S4*B*). Hemin was used to increase intracellular heme levels, the endogenous activator of

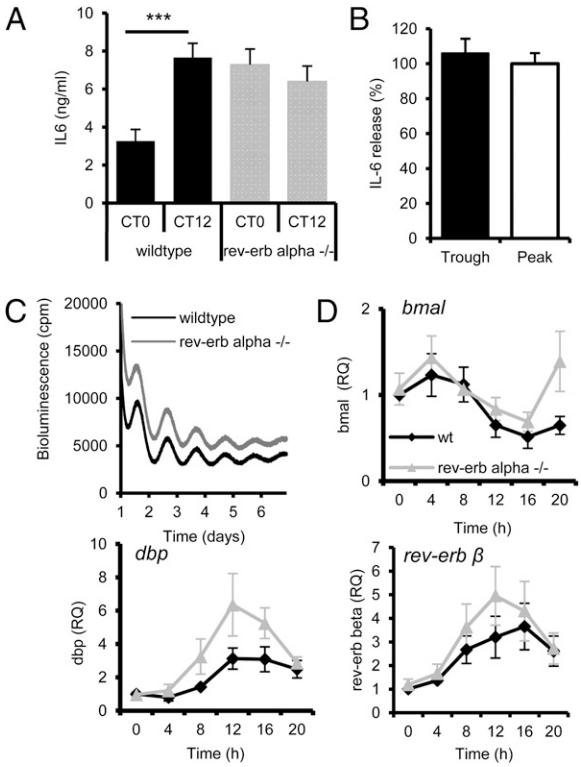

Fig. 3. Loss of gating in *rev-erbα*$^{-/-}$ mice. (*A*) IL-6 release after in vivo LPS does not differ between CT0 and CT12 administration in *rev-erbα*$^{-/-}$ mice, unlike WT littermates (n = 6–7, ANOVA and Bonferroni). (*B*) PECs isolated from *rev-erbα*$^{-/-}$ mice show loss of a gated response to LPS (n = 3–4). (*C*) Isolated PECs from *rev-erbα*$^{-/-}$ mice on a PER2::luc background show circadian oscillations (period 24.1 ± 0.4) comparable to WTs (period 23.7 ± 0.4; n = 4). (*D*) Circadian profiling shows that *bmal*, *rev-erbβ*, and *dbp* mRNA expression in PECs remains circadian despite the absence of *rev-erbα*; transcripts are reported relative to time 0 in WT mice (n = 3).

REV-ERB (14), and like GSK4112, hemin significantly suppressed *il6* induction after LPS (Fig. 4*D*). Control experiments confirmed that neither hemin nor GSK4112 treatment alone affected *il6* expression. To confirm REV-ERBα dependency, THP-1 cells were transduced with shRNA to suppress endogenous *rev-erbα*. Knockdown was validated by qPCR (showing 75–80% knockdown; Fig S4*C*). Suppression of *rev-erbα* expression (using shRNA) increased *il6* (but not *il8*) mRNA response to LPS, and in the absence of *rev-erbα*, the inhibitory effects of GSK4112 on *il6* expression were abolished (Fig. 4 *E* and *F*). Thus, we demonstrate REV-ERBα repression of IL-6 in three human macrophage cell models, which can be regulated by endogenous and synthetic REV-ERB ligands.

REV-ERB Ligand Modulation of Innate Immune Responses. To explore the wider spectrum of action of GSK4112, we mapped responses of LPS-stimulated human MDMs by Affymetrix gene array; this revealed a significant number of LPS-activated genes regulated by the ligand, and, as expected, the majority was repressed. Ontological profiling identified 102 significant terms linked to the repressed genes with the majority linked to immune response (Benjamini $P < 1 \times 10^{-14}$), included within were a large number of genes implicated in inflammatory diseases. Our initial studies in mouse and human macrophage cells identified IL-6, CCL2, IL-12(p40), and CCL5 as common REV-ERB target genes in both species. This array identified additional chemokine (*cxcl11*, *cxcl6*) and cytokine (*il19*) genes that were repressed by GSK4112 (interestingly, the anti-inflammatory cytokine *il10* was also repressed) (Fig. S5*A*). These results were validated by qPCR, to confirm ligand-dependent suppression of each (Fig. 5). As a control, *il8* remained nonresponsive. Bioinformatic analysis (DiRE program) of the regulated genes for conserved response elements revealed significant enrichment in several predicted transcription factor binding sites, including NF-κB, and LXR (Fig. S5*B*). Importantly, the LXR response element is also recognized by REV-ERBα.

In view of the temporal differences between *rev-erbα* expression and the peak of IL-6 response to LPS, it is important to consider that REV-ERBα may not directly regulate cytokine responses; instead, an indirect mechanism may be in place whereby REV-ERBα represses a repressor. One candidate repressor is nuclear factor IL-3 regulated (NFIL3), which shows circadian expression and can be REV-ERB regulated (15, 16). NFIL3 plays a role in the immune response (17–19) and is a direct regulator of murine *il12(p40)* and *il6* (20). Whether other murine-gated cytokines identified here are under NFIL3 control is unknown, and this represents an interesting avenue to explore. However, in the specific instance of human macrophage cells, the REV-ERB ligand did not regulate *nfil3*, whereas *il6* was significantly repressed (Fig. S4*D*), hence other intermediary repressors may be involved.

Discussion

Our studies in mice reveal that the endotoxin-driven inflammatory response is gated in magnitude by the circadian clock. Specifically, a subset of cytokines, including the prominent proinflammatory cytokine IL-6, is regulated in a time-of-day–dependent manner. This selective gating mechanism was observed in vivo and persisted in cultured immune cells. Using genetically modified mice to ablate the macrophage clockwork, we eliminated the in vitro rhythmic cytokine release in response to LPS, and this was translated into a loss of overall circadian cytokine responsiveness in vivo. Thus, our studies identify the macrophage as key to the rhythmic response to systemic administration of LPS. In *LysM-bmal*$^{-/-}$ mice, cytokine responses at night (active phase) were of similar magnitude to those of WT mice, whereas in the subjective day (rest phase) responses were augmented to levels comparable to those observed in the active phase of WT mice. Therefore, it appears that clock action in macrophages serves as a selective rhythmic repressor of cytokine induction. This finding is supported by previous studies showing circadian control of CCL2 and IL-6 in vivo and in vitro (5, 21). The conditional targeting strategy we used does not permit formal dissociation of direct circadian-mediated effects (loss of circadian oscillations per se) from pleiotropic actions of specific clock genes on inflammatory pathways. However, using a global REV-ERBα knockout model, we demonstrated similar loss of gated cytokine responses, despite maintenance of strong circadian rhythmicity in macrophages. Together, these data implicate the circadian clockwork, and REV-ERBα specifically, in gating the innate immune response.

Recent studies have shown that REV-ERBα mediates clock control of multiple cellular metabolic pathways, including hepatic lipid metabolism and regulation of sterol regulatory element-binding protein (22). Circadian homeostasis, metabolism, and immune responses share common pathways (23), and our data extend the role of REV-ERBα beyond metabolism to include innate immunity. The recent development of the REV-ERB ligand GSK4112 significantly enhances our ability to dissect the role of this nuclear receptor in normal tissue physiology. Recent studies show GSK4112 to act on REV-ERBβ (24); however, our data strongly suggest REV-ERBα as the key interface with innate immunity. Ligand control of REV-ERB action adds a level of complexity to the regulation of inflammatory responses, because the synthesis and degradation of the endogenous ligand heme are regulated both by inflammation and circadian factors (25, 26).

Many human inflammatory diseases have diurnal variation in severity; however, the mechanisms underpinning this physiology remain poorly understood. In RA a robust circadian rhythm in serum IL-6 concentration strongly tracks changes in inflammation severity (2), and IL-6 is known to play a direct role in this

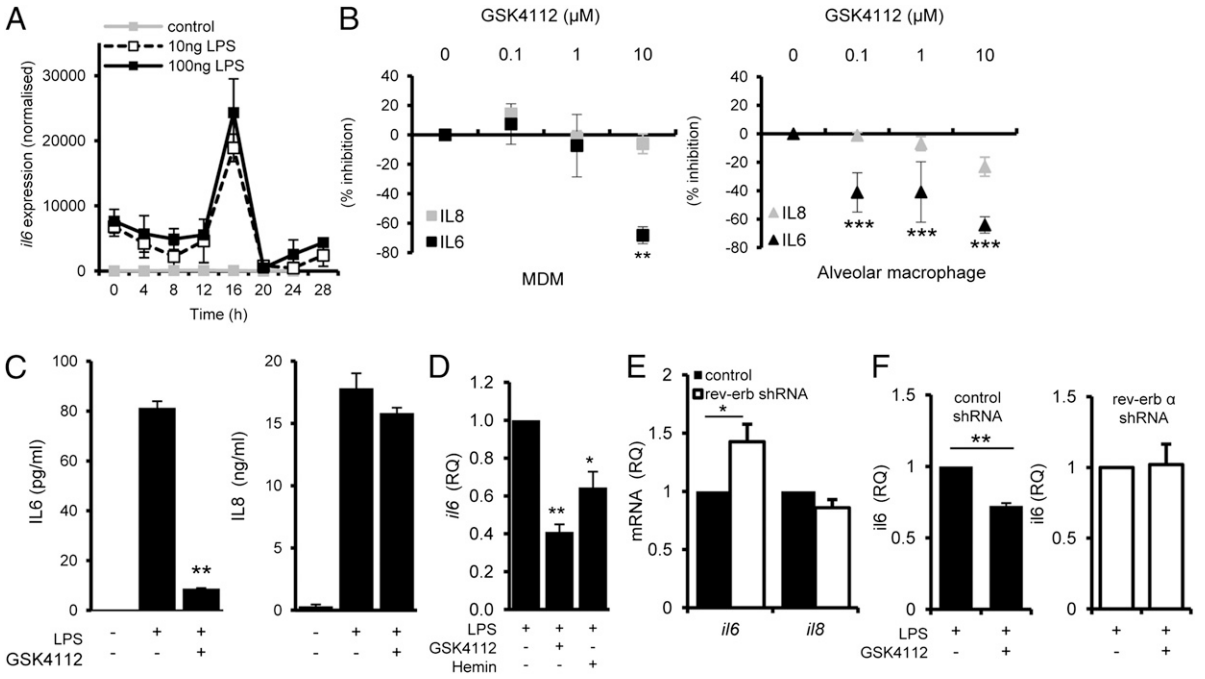

Fig. 4. Circadian gating in human cells. (*A*) In human MDMs, *il6* mRNA response to LPS peaks 16 h after serum synchronization (values are mean ± SD, $n = 4$). (*B*) In response to LPS, IL-6 release (but not IL-8) is inhibited by the REV-ERB ligand GSK4112 in both MDMs ($n = 3$) and primary alveolar macrophages ($n = 18$). Cells were incubated with LPS and GSK4112 for 16 h before harvest (one-way ANOVA, post hoc Bonferroni). (*C*) IL-6 protein (but not IL-8) expression by THP-1 cells in response to LPS is significantly attenuated by application of GSK4112 (one-way ANOVA, post hoc Bonferroni). (*D*) *il6* mRNA expression after LPS application is reduced by both GSK4112 and hemin in THP-1 cells ($n = 3$, one-way ANOVA, post hoc Bonferroni); transcript abundance is reported relative to LPS alone. (*E*) shRNA knockdown of *rev-erbα* in THP-1 cells increases *il6* (but not *il8*) mRNA after LPS challenge compared with controls ($n = 3$, *t* test). (*F*) Knockdown of *rev-erbα* abolishes the inhibition of *il6* mRNA abundance seen with GSK4112 ($n = 3$, *t* test).

chronic inflammatory disease (27–29). Using endogenous and synthetic REV-ERB ligands, we showed regulation of IL-6 and other human proinflammatory cytokines and chemokines in primary human macrophages and a myelomonocytic cell line; several of these were also REV-ERBα regulated in the mouse. This cytokine set includes CCL2, which is genetically linked to asthma and has a strong circadian component. Strikingly, IL-8, known not to show a diurnal variation in human serum (2), showed no REV-ERBα response. Throughout our studies, IL-6 was a consistent target, and suppression of *rev-erbα* by shRNA resulted in exaggerated IL-6 responses to LPS, suggesting tonic inhibitory action. Furthermore, this *rev-erbα* knockdown established receptor specificity for the ligand effects.

Previous studies have shown that REV-ERBα can regulate human *tlr4* expression (10), but the discordant regulation of IL-6 and IL-8 (both TLR4 activated) suggest a selective mode of action. In support, our data show that ligand modulation of LPS responses is not dependent on altered *tlr4* expression. Recent studies have shown that inflammatory responses are regulated by NCoR and silencing mediator of retinoic acid and thyroid hormone receptor corepressor complexes, which are required for the anti-inflammatory actions of LXR (30, 31). REV-ERBα–mediated repression acts via recruitment of NCoR, and REV-ERB shares a common DNA binding motif with the nuclear receptors LXR and peroxisome proliferator-activated receptor. Intriguingly, LXR binding sites were identified as overrepresented in ligand-regulated genes, and some REV-ERB target genes, including CCL2 and CXCL1, are specifically repressed by NCoR, whereas IL-8 (not regulated by REV-ERBα) is not (31).

Daily risk of infection is likely to be a direct consequence of activity and feeding. Our studies show that during the rest phase, the cytokine response is on the rise, peaking at the transition between rest and activity. Our data are compatible with the hypothesis that when infection is most likely, the cytokine response is amplified to offer enhanced protection. An apparent paradox of our data relates to the expression profiles of *rev-erbα* and the phasing of endotoxin responses. We observed peak mRNA expression at the time of maximal LPS responses, but our gene knockout studies in mouse, and knockdown studies in human cells, suggest an inhibitory action of REV-ERBα on IL-6 expression. This temporal displacement between the peak of REV-ERBα expression and the peak of IL-6 repression requires some explanation. The dynamics of cellular REV-ERB protein

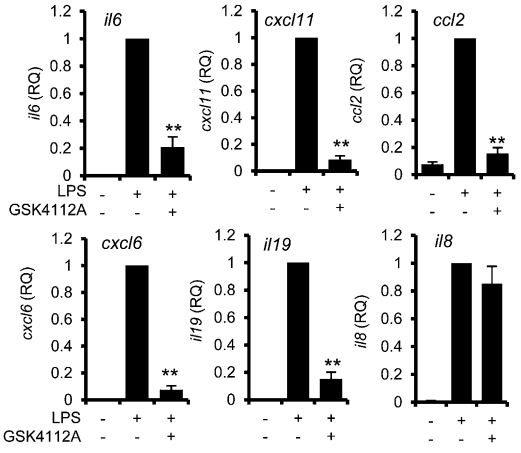

Fig. 5. Confirmation of the microarray study in human primary MDMs. Transcription of a selection of cytokines, but not *il8*, after LPS challenge is attenuated by coapplication of the REV-ERB ligand GSK4112 ($n = 6$, one-way ANOVA, post hoc Bonferroni); transcript abundance is reported relative to LPS-treated cells.

turnover are yet to be defined, and there may be a temporal delay in transcriptional repression dependent on ligand binding or posttranslational modification. However, it is also possible that REV-ERBα operates via an indirect mechanism involving action on intermediate repressors, although one such candidate (NFIL3) does not appear to be involved in human macrophage responses.

In summary, we show that REV-ERBα acts as a nodal output of the clock, linking cellular circadian timers with innate immune responses. Using three sources of human macrophages, we show that innate immune responses are pharmacologically tractable in a REV-ERBα–dependent manner. Our data reveal common and selective patterns of regulation of proinflammatory cytokines in man and mouse. A challenge for the future relates to defining the biochemical mechanisms through which the circadian clockwork selectively regulates components of the innate immune response.

Methods

Mouse Lines. All experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act of 1986. *Rev-erbα*$^{-/-}$ mice (12) were provided by Ueli Schibler (University of Geneva). *Bmal*$^{flox/flox}$ mice (32) were bred with LysM$^{cre/+}$ mice (33) to target *bmal* for deletion in cells of a myeloid lineage (including monocytes, mature macrophages, and granulocytes; *SI Methods*). Both strains were crossed onto a PER2::luc background (8). Circadian activity was monitored using wheel running. For in vivo LPS challenge, mice were individually housed in 12:12 light/dark cycles. After 24 h of constant darkness, LPS (or saline) was administered i.p. (0127:B8, 1 mg/kg) at CT0 or CT12. At 4 h after treatment, serum cytokines were quantified using suspension array technology (Bio-Plex System; BioRad). In separate experiments, mice were administered LPS at CT0 or CT12; 30 min later, PECs were collected via lavage and the RNA extracted.

Cell Culture. PECs were isolated, resuspended in RPMI, and plated out. For gene expression time courses, cells were synchronized (50% FBS, 1 h) and RNA collection began 20 h later (defined as time 0), every 4 h. Human MDMs were obtained from buffy coats acquired from the National Blood Transfusion Service (UK).

In Vitro LPS Challenge and Application of REV-ERB Ligands. PECs were challenged with LPS (100 ng/mL) for 4 h and the supernatant collected. PECs from PER2::luc mice were isolated in parallel and run under the photomultiplier system to report PER2 activity. Human cells were treated with LPS in the absence of presence of the REV-ERB ligand (10 μM unless otherwise stated) or hemin (1 μM). GSK4112 was synthesized by GlaxoSmithKline. Cytokine analysis was carried out by ELISA.

Affymetrix Gene Array. Human MDMs were stimulated with LPS plus or minus GSK4112, and RNA extracted, amplified, reverse transcribed, and hybridized onto an Affymetrix U133 plus 2.0 chip.

Data Analysis. Unless stated, values are presented as mean ± SEM. Statistical analysis was performed using SPSS 16.0. Two group comparisons were performed using Student *t* test, and for more than two groups by ANOVA and post hoc Bonferroni *t* test. Significance values were $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.005$. Cosinor analysis was performed using Cosinor.exe version 2.3 (Roberto Refinetti, University of South Carolina, Salkehatchie, SC). RAP software analysis was used to analyze period of Per2::luc cells.

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