Susceptibility rhythm to bacterial endotoxin in myeloid clock-knockout mice

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Abstract

Local circadian clocks are active in most cells of our body. However, their impact on circadian physiology is still under debate. Mortality by endotoxic (LPS) shock is highly time-of-day dependent and local circadian immune function such as the cytokine burst after LPS challenge has been accounted for the large differences in survival. Here, we investigate the roles of light and myeloid clocks on mortality by endotoxic shock. Strikingly, mice in constant darkness (DD) show a three-fold increased susceptibility to LPS as compared to mice in light-dark conditions. Mortality by endotoxic shock as a function of circadian time is independent of light-dark cycles as well as myeloid CLOCK or BMAL1 as demonstrated in conditional knockout mice. Unexpectedly, despite the lack of a myeloid clock these mice still show rhythmic patterns of pro- and anti-inflammatory cytokines such as TNFα, MCP-1, IL-18 and IL-10 in peripheral blood as well as time-of-day and site dependent traffic of myeloid cells. We speculate that systemic time-cues are sufficient to orchestrate innate immune response to LPS by driving immune functions such as cell trafficking and cytokine expression.

Introduction

Timing of immune-functions is crucial for initiating, establishing, maintaining and resolving immune-responses. The temporal organization of the immune system also applies for daily recurring tasks and may even help to anticipate times of environmental challenges. In humans, many parameters and functions of the immune system display diurnal patterns [43], which impact on disease severity and symptoms [13]. While the concepts of chronobiology are increasingly acknowledged in life-science and medicine [5], a deep comprehension of how time-of-day modulates our physiology in health and disease is still lacking.

The fundamental system behind the time-of-day dependent regulation of an organism, its behavior, physiology and disease is called the circadian clock. In mammals, this clock is organized in a hierarchical manner: a central pacemaker in the brain synchronized to environmental light-dark cycles via the eyes and peripheral clocks receiving and integrating central as well as peripheral (e.g. metabolic) time information. Both central and peripheral clocks are essentially identical in their molecular makeup: Core transcription factors form a negative feedback loop consisting of the activators, CLOCK and BMAL1, and the repressors, PERs and CRYs. Additional feedback loops and regulatory factors amplify, stabilize and fine-tune the cell intrinsic molecular oscillator to achieve an about 24-hour (circadian) periodicity of cell- and tissue-specific clock output functions [3].

Circadian patterns of various immune-functions have been reported in mice [43] and other species [24,30,48] including cytokine response to bacterial endotoxin and pathogens, white blood cell traffic [7, 38, 44] and natural killer cell activity [2]. Cell-intrinsic clocks have been described for many leukocyte subsets of lymphoid as well as myeloid origin, including monocytes/macrophages [28]. Furthermore, immune-cell intrinsic clocks have been connected to cell-type specific output function such as the TNFα response to LPS in macrophages [28].

Sepsis is a severe life threatening condition with more than 31 million incidences per year worldwide [19]. Mouse models of sepsis show a strong time-of-day dependency in mortal-

ity rate when challenged at different times of the day [12, 17, 18, 23, 26]. Most studies agree about the times of highest (around *Zeitgeber time* [ZT]8 - i.e. 8 hours past lights on) or lowest (around ZT20) mortality across different animal models and investigators [17, 18, 23, 26]. The fatal cascade in the pathomechanism of endotoxic shock is initiated by critical doses of LPS recognized by CD14 bearing monocytes/macrophages leading to a burst of pro-inflammatory cytokines. A viscous cycle of leukocyte recruitment, activation and tissue factor (III) expression triggers disseminated intravascular coagulation and blood pressure decompensation and final multi-organ dysfunction [37].

Here, we investigate the impact of light-dark cycles and local myeloid clocks on time-of-day dependent survival rates in endotoxic shock using conditional clock-knockout mouse models. We show that peripheral blood cytokine levels as well as mortality triggered by bacterial endotoxin depend on time-of-day despite a functional clock knockout in myeloid cells. Our work thus challenges current models of local regulation of immune responses.

Results

Time-of-day dependent survival in endotoxic shock

Diurnal patterns of LPS-induced mortality (endotoxic shock) have been reported numerous times in different laboratory mouse strains [23, 36, 44]. To address the question, whether time-of-day dependent susceptibility to LPS is under control of the circadian system rather than being directly or indirectly driven by light, we challenged mice kept either under light-dark conditions (LD 12:12) or in constant darkness (DD) at four different times during the cycles. As expected from previous reports, survival of mice housed in LD was dependent on the time of LPS injection, being highest during the light phase and lowest at night (Fig. 1*A*).

Surprisingly, mice challenged one day after transfer in constant darkness using the same dose showed a more than 60% increase in overall mortality compared to mice kept in LD (Fig. 1*A*). Furthermore, time-of-day dependent differences in mortality were much less pronounced under these conditions.

To discriminate, whether constant darkness alters overall susceptibility to LPS leading to a ceiling effect rather than eliminating time-dependent effects, we systematically reduced LPS dosage in DD conditions. By challenging the mice at four times across the day we determined the half-lethal dose of LPS in constant darkness (Fig. 1*B*). As suspected, mice in DD showed a 3-fold increased susceptibility to LPS-induced mortality. For circadian rhythm analysis, we extended the number of time points at approximately half-lethal doses both in LD and DD groups, respectively. This revealed diurnal/circadian patterns in mortality rate in LD (p-value=0.06) as well as in DD conditions (p-value=0.001) (Fig. 1*C*) demonstrating that the circadian system controls susceptibility to LPS. In addition, this susceptibility is overall increased in constant darkness.

Circadian cytokine response upon LPS challenge in mice

We and others have recently found that cells in the immune system harbor self-sustained circadian oscillators, which shape immune functions in a circadian manner [25, 28]. Pro-inflammatory responses in murine *ex vivo* macrophage culture [4, 28] are controlled by cell-intrinsic clocks and are most prominent during the day and lowest during the activity phase. Furthermore, pro-inflammatory cytokines such as TNFα, IL-1α/β, IL-6, IL-18 as well as MCP-1 have been linked to the pathomechanism of endotoxic shock [1, 22, 31, 35, 49].

Thus, we hypothesized that the cytokine response in LPS-challenged mice has a time-of-day dependent profile, which might govern the time-of-day dependent mortality rates in the endotoxic shock model. Indeed, plasma of mice collected two hours after administratioin of half-lethal doses of LPS (either in LD (30mg/kg) or DD (13mg/kg) conditions at various times during the day), exhibited an up to two-fold time-of-day difference in absolute cytokine concentrations (Fig. 1*D*, and *E* for amplitude and phase informatin as determined by sine fit). In animals kept in LD, TNFα, showed highest levels around ZT8, IL-18 levels peaked at ZT18 and IL-12 as well as the anti-inflammatory cytokine IL-10 had their peak-time around ZT14. Interestingly, cytokine profiles from DD mice differed substantially from LD profiles: the peak-times of IL-12 was phase-advanced by 6 hours, CXCL5 completely reversed its phase, whereas IL-18 remained expressed predominantly in the night. Taken together, cytokine profiles of LPS-challenged mice parallel endotoxic shock-induced mortality patterns,

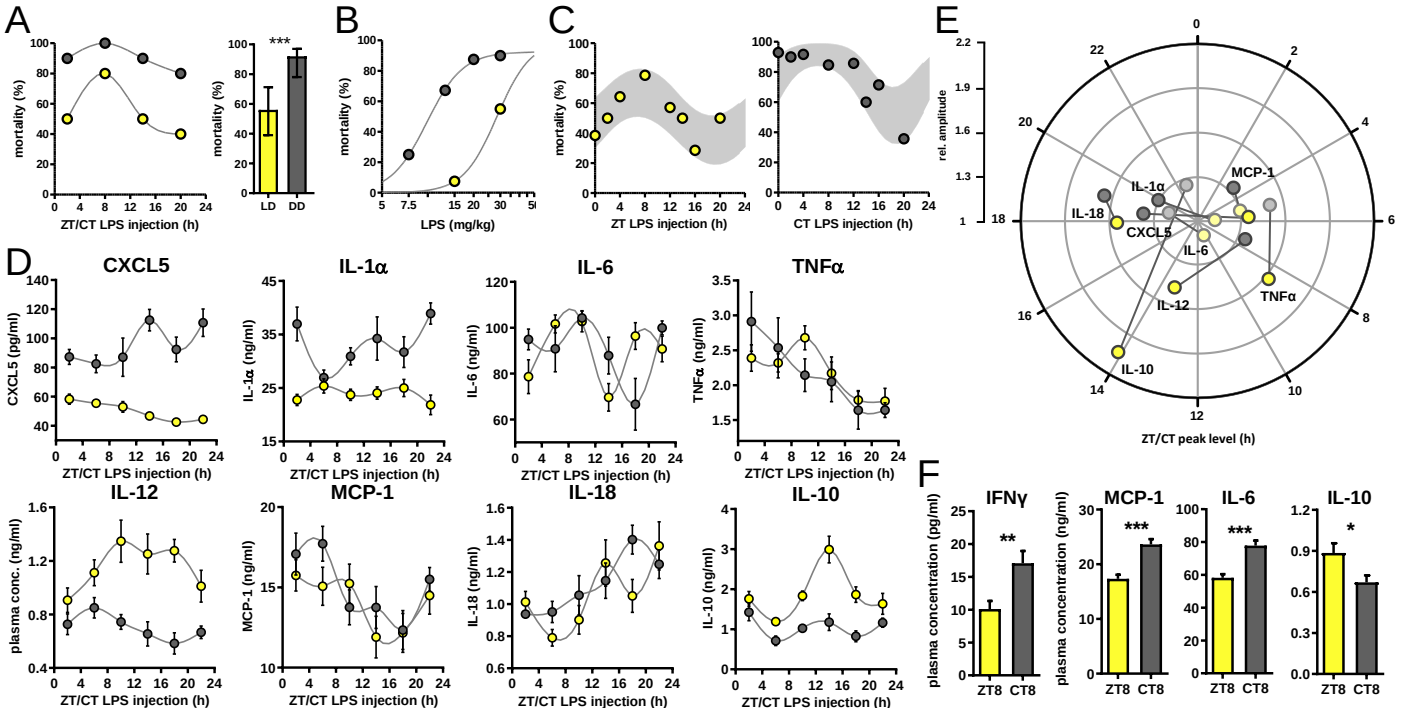

Figure 1. Time-of-day dependent mortality in LPS treated mice is controlled by the circadian system and light conditions. A) LPS (30mg/kg, *i.p.*) induced mortality in C57Bl/6 mice (n=10 per time point) kept either in LD 12:12 (yellow) or in DD (grey). Left graph: single time points; right graph: mean mortality of LD or DD light condition. Error bars represent 95% confidence intervals (n=40 per group, *** $p<0.001$). **B)** LPS dose-mortality curves of mice challenged at 4 time points in LD versus DD (overall n=40 mice per dose). Gray lines were calculated by fitting an allosteric model to each group. **C)** Mice (n=10-14 per time point) were challenged with half-lethal doses of LPS (30 mg/kg, *i.p.*, for mice kept in LD (left panel) or 13mg/kg, *i.p.*, for mice kept in DD (right panel). Mortality was assessed 60 hours after LPS injection. To perform statistical analyses, mortality rates were transformed to probability of death in order to compute sine fit using logistic regression and F-test (LD, p=0.06; DD, p=0.001; gray shaded areas indicate 95% confidence intervals. **D) and E)** Time-of-day dependent cytokine profiles in peripheral blood of C57Bl/6 mice (n=10 per time point) challenged with half-lethal doses of LPS (30mg/kg, *i.p.*, for mice kept in LD (yellow) or 13mg/kg, *i.p.*, for mice kept in DD (gray)) and sacrificed 2 hours later. **E**) Relative amplitudes and phases of cytokines shown in D). Light-colored circles represent non-significant circadian rhythms (p-value>0.05) as determined by non-linear least square fit and consecutive F-test (see also Methods section). **F)** Cytokine levels in peripheral blood from mice (n=10 per time point) challenged with LPS (13mg/kg, *i.p.*) at either ZT8 (LD) or CT8 (DD) conditions (T-test, *** $p<0.001$, ** $p<0.01$, *$p<0.05$).

although most circadian cytokines show variable phase relations between free-running and entrained conditions (Fig. 1*E*).

Next, we investigated, whether the increased overall mortality in DD was correlated with an increased pro-inflammatory cytokine response. We thus injected mice at either ZT8 (mice kept in LD) or CT8 (mice kept in DD) with the same dose of LPS (13mg/kg) and took blood samples two hours later. IFNγ, MCP-1, IL-6 and the anti-inflammatory cytokine IL-10 showed significantly altered levels between mice kept in LD or DD (Fig. 1*F*), suggesting that in DD conditions the sensitivity to endotoxin is increased leading to enhanced pro-inflammatory cytokine secretion and subsequently increased mortality.

Dispensable role of myeloid clocks in circadian endotoxin reactivity

Local clocks are thought to play important roles in mediating circadian modulation of cell- and tissue-specific functions [47]. In fact, depletion of local immune clocks has been shown to disrupt circadian patterns of tissue function [9,14,20,21,39]. To test whether local clocks in cells of the innate immune system are responsible for circadian time dependency in the response to bacterial endotoxin, we challenged myeloid lineage *Bmal1*-knockout mice (*LysM-Cre*$^{+/+}$ x *Bmal1*$^{flox/flox}$, hereafter called myBmal-KO) with half-lethal doses of LPS at various times across the circadian cycle. These mice lack physiological levels of Bmal1 mRNA and protein in cells of myeloid origin and have been characterized elsewhere [21]. To our surprise, mortality of these mice was still dependent on circadian time of LPS administration (Fig. 2*A*) indicating that a functional circadian clock in myeloid cells is not required for time-of-day dependent LPS-sensitivity. However, the overall susceptibility to LPS decreased two-fold compared to wild-type mice (Fig. 2*B*) suggesting that BMAL1 levels in myeloid cells directly

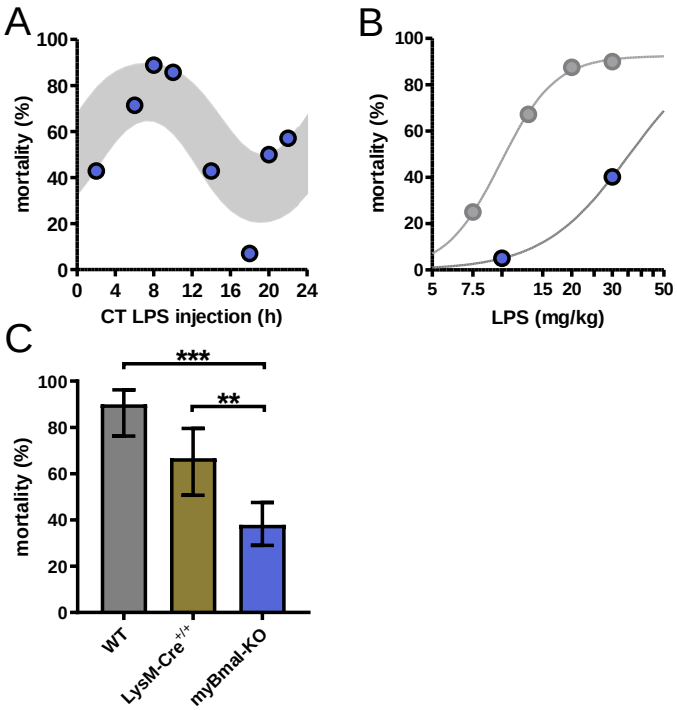

Figure 2. myBmal-KO mice show time-of-day dependent and increased susceptibility to LPS. A) Circadian mortality in myBmal-KO mice. Mice (n=10-14 per group) were challenged with half-lethal doses of LPS (30mg/kg, *i.p.*) at indicated time points. Mortality was assessed 60 hours after LPS injection. Statistics were performed as in Fig. 1*C*, (p=0.0009; gray shaded area indicates 95% confidence interval). **B)** LPS dose-mortality curves of mice challenged at 4 time points in constant dark conditions. About 3-fold decrease of susceptibility to LPS in myBmal-KO mice (blue circles) as compared to wild-type mice (gray circles – replotted from Fig. 1*B*) kept in DD. Gray lines were calculated by fitting an allosteric model to each group. **C)** Reduced mean mortality in myBmal-KO mice (n=84) compared to control strains LysM-Cre$^{+/+}$ or C57Bl/6 (wild-type, n=40). Error bars represent 95% confidence intervals (n=40 per group, *** $p<0.001$, ** $p<0.01$).

or indirectly modulate susceptibility towards LPS. The latter effect could only in part be attributed to genetic background (note decreased susceptibility to LPS in *LysM-Cre*$^{+/+}$ control mice (Fig. 2*C*)) together arguing for a tonic rather than temporal role of myeloid BMAL1 in regulating LPS sensitivity.

Despite its essential role for circadian clock function, BMAL1 has been linked with a number of other non-rhythmic processes such as adipogenesis [45], sleep regulation [16] and cartilage homeostasis [15]. Thus, we asked whether the decreased susceptibility to LPS observed in myBmal-KO mice was due to non-temporal functions of BMAL1 rather than to the disruption of local myeloid clocks. CLOCK, like its heterodimeric binding partner BMAL1, has also been shown to be an indispensable factor for peripheral clock function [11]. If non-temporal outputs of myeloid clocks rather than gene specific functions of myeloid BMAL1 controls the susceptibility to LPS, a depletion of CLOCK should copy the myBmal-KO phenotype. We therefore generated conditional, myeloid lineage specific Clock-KO mice (hereafter called myClock-KO). As in myBmal1-KO mice, the expression of Cre recombinase is driven by a myeloid specific promoter (LysM) consequently leading to excision of LoxP flanked exon 5 and 6 of the clock gene [10]. As expected, the expression of *Clock* mRNA and protein was substantially reduced in peritoneal cavity cells but was normal in liver (Fig. 3*A*-*B*).

To investigate, whether clock gene rhythms were truly abolished in myClock-KO mice, we harvested peritoneal macrophages from myClock-KO or control mice (LysM-Cre) in regular 4-hour intervals over the course of 24 hours. Rhythmicity of *Bmal1*, *Cry1*, *Cry2*, *Dbp*, *Npas2* and *Nr1d1* mRNA levels was essentially eliminated, while a low amplitude rhythmicity was detected for *Per1* and *Per2* mRNA (Fig. 3*C* and *D*). Moreover, circadian oscillations were disrupted in peritoneal cavity cells (mainly macrophages and B-cells) from myClock-KO mice, but not in tissue explants from SCN or lung (Fig. 3*E*).

Given the disruption of rhythmicity in myClock-KO myeloid cells, we asked whether

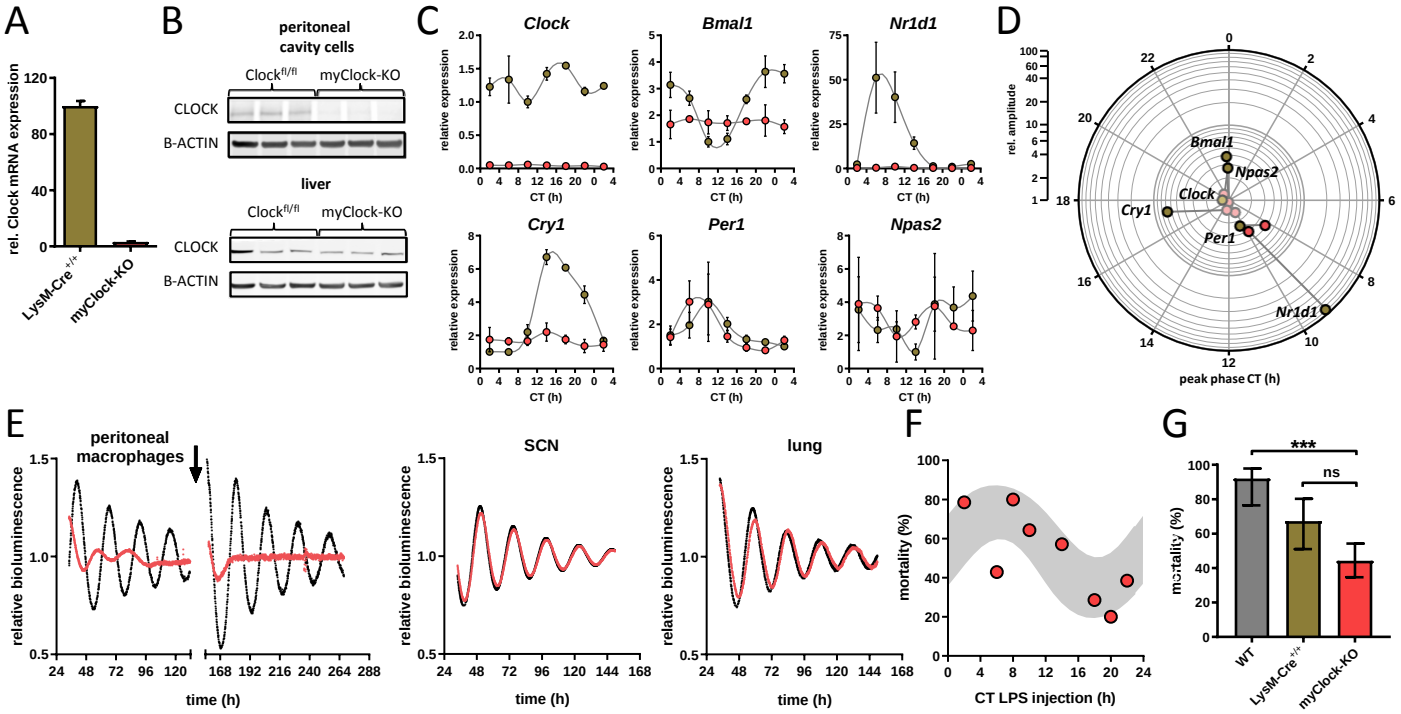

Figure 3. Conditional myClock-KO mice show circadian pattern in mortality by endotoxic shock. A) and B) Reduced levels of *Clock* mRNA and protein in myeloid lineage cells of myClock-KO mice. **A)** Mean values of normalized mRNA expression values of time-series shown in C) (n=21 mice per condition, $p<0.0001$, t-test). **B)** Protein levels by immunoblot in peritoneal cavity cells and liver of myClock-KO and *Clock*$^{fl/fl}$ control mice (n=3). **C)** Relative mRNA levels of selected clock genes in peritoneal macrophages from LysM-cre$^{+/+}$ (brown circles) or myClock-KO (red circles) mice at indicated circadian times. Phase and amplitude information are depicted in D) as analyzed by Chronolyse. Non-significant circadian expression ($p>0.05$) are depicted in light red (myClock-KO) or light brown (LysM-cre control). **E)** Representative bioluminescence recordings of peritoneal macrophages, SCN or lung tissue from myClock-KO or wild-type mice crossed with PER2:Luc reporter mice (color coding as before). Black arrow indicates time of re-synchronization by dexamethason treatment (detrended data). **F)** Circadian pattern in endotoxic shock mortality despite deficiency of CLOCK in myeloid lineage cells. Mice (n=10-14 per time point) were challenged with half-lethal doses of LPS (30mg/kg, *i.p.*) at indicated time points. Mortality was assessed 60 hours after LPS injection. Statistic were performed as in Fig 1*C* (p=0.005, gray shaded area indicates 95% confidence interval). **G)** Reduced mean mortality (at 30mg/kg LPS) in mice deficient of myeloid CLOCK (n=84) compared to control strains LysM-Cre$^{+/+}$ (n=40) or C57Bl/6 (wild-type, n=40). First two bars where re-plotted from Fig. 2*C*. Error bars represent 95% confidence intervals (n=40 per group, ns $p>0.05$, p*** $p<0.001$.

CLOCK in myeloid cells is required for time-of-day dependent mortality in endotoxic shock. To this end, we challenged myClock-KO mice at various times during the circadian cycle with half-lethal doses of LPS. Again, mortality in these mice was significantly time-of-day dependent (Fig. 3*F*). As in myBmal1-KO mice, myClock-KO mice showed strongly reduced susceptibility to LPS compared to wild-type mice (Fig. 3*G*). Taken together, our data unequivocally show that myeloid clockworks are dispensable for the time-of-day dependency in endotoxic shock. In addition, decreased overall susceptibility suggest a non-temporal, sensitizing role of myeloid CLOCK/BMAL1 in the regulation of endotoxic shock.

Circadian cytokine response in myClock-KO mice

Our initial hypothesis was built on the assumption that a time-of-day dependent cytokine response determines the outcome in endotoxic shock. Previous results from us and others [21,25,28] suggested that local myeloid clocks govern the timing of the pro-inflammatory cytokine response. However, circadian mortality profiles in LPS-challenged myeloid clock-knockout mice (Fig. 2*A* and 3*F*) led us to question this model: The circadian cytokine response in plasma is either independent of a myeloid clock or the circadian mortality by endotoxic shock does not require a circadian cytokine response.

To test these mutually not exclusive possibilities, we challenged myClock-KO mice with half-lethal doses of LPS in regular 4-hour intervals over the course of one day. Unexpectedly, cytokine levels in plasma, collected two hours after LPS administration still exhibited circadian patterns for TNFα, IL-18, IL-10 (Fig. 4*A*) (p-values=0.046, 0.001 and 0.009, respectively), very similar to those observed in wild-type animals (Fig. 4*B*). Other cytokines remained below statistical significance threshold for circadian rhythmicity tests (IL-1α) or

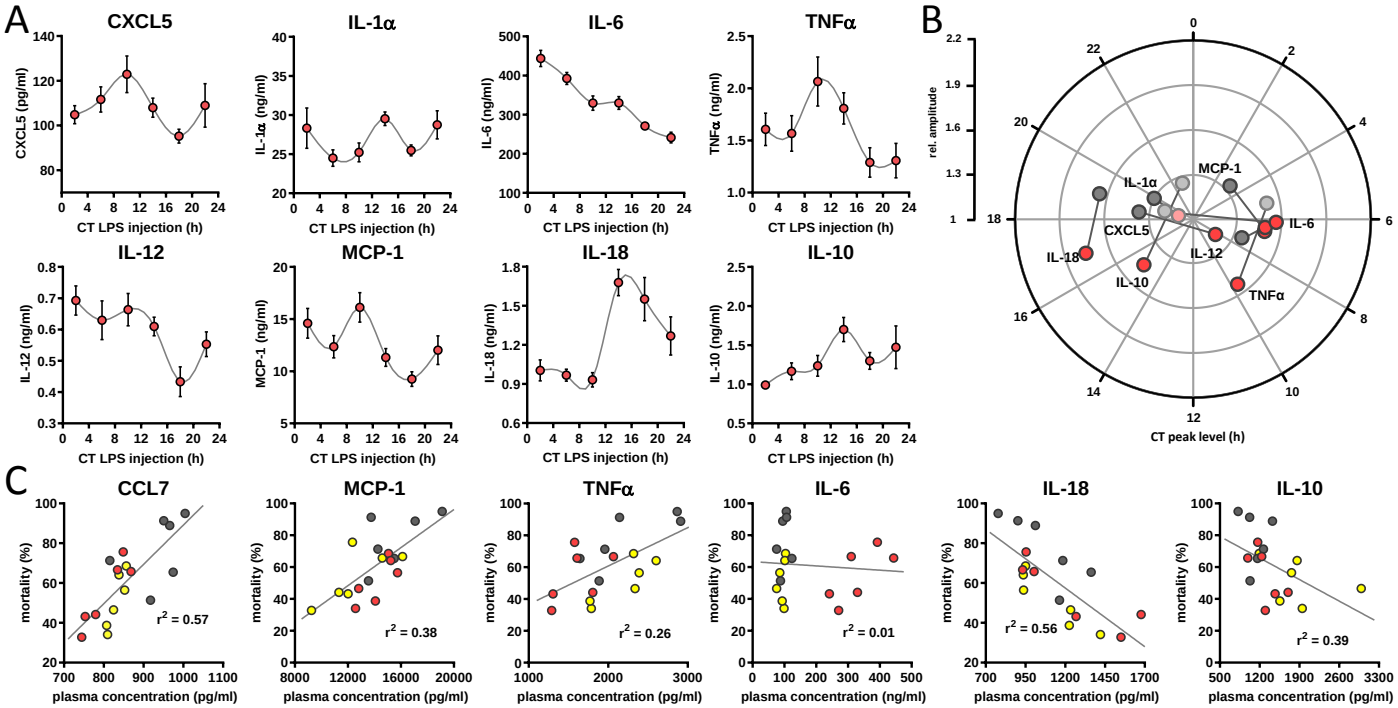

Figure 4. Circadian time dependent cytokine levels in plasma of myClock-KO mice. A) Plasma cytokine levels in myClock-KO mice kept in constant darkness, two hours after injection of 30mg/kg LPS. Data represent mean values ± SEM (n=14 per time point). **B)** Polar plot showing amplitude and phase distribution of pro- and anti-inflammatory cytokines from A), red circles and wild-type DD (Fig. 1*E*). Light circles indicate non-significant (p-values>0.05, non-linear least square fit statistics by ChronoLyse) circadian abundance. **C)** Overall correlation of cytokine levels with mortality independent of time-of-day of LPS injection and mouse model. Colors indicate data source (wild-type, LD - yellow; wild-type, DD - gray; myClock-KO, DD - red; linear regression - gray line; statistics: spearman correlation).

displayed large trends within this period of time (IL-6). These data suggest that a LPS-induced circadian cytokine response does not depend on a functional circadian clock in cells of myeloid origin. Thus, circadian cytokine expression might still be responsible for time-of-day dependent mortality in endotoxic shock.

To identify those cytokines, whose levels best explain mortality upon LPS challenge, we correlated cytokine levels and mortality rate for all conditions - independent of time-of-day or mouse strain - in a linear correlation analysis. Levels of CCL7, MCP-1 and TNFα showed strong positive correlation with mortality (p-values=0.0003, 0.0065, 0.0319, respectively). Others, such as IL-10 and IL-18 correlated negatively (p-values=0.0054 and 0.0004, respectively) (Fig. 4C). Interestingly, while TNFα and IL-10 are well known factors in the pathomechanism of endotoxic shock, CCL7, MCP-1 and protective effects of IL-18 have not been reported in this context.

Together, our data suggest that local circadian clocks in myeloid lineage cells are dispensable for time-of-day dependent plasma cytokine levels upon LPS challenge. However, where does time-of-day dependency in endotoxic shock originate instead?

Persistent circadian traffic in myeloid clock-knockout mice

Circadian patterns in immune cell trafficking and distribution have been recently reported and linked to disease models and immune functions [14, 28, 39]. Similarly, homing and release/egress of hematopoetic stem cells (HSPCs), granulocytes, and lymphocytes to bone marrow and lymph nodes, respectively, have been shown to vary in a time-of-day dependent manner requiring the integrity of an immune-cell intrinsic circadian clock [7, 14, 38, 44]. Thus, we asked, whether circadian traffic of myeloid cells can be associated with mortality rhythms in our endotoxic shock model.

To test this, we measured the number of immune cells in various immunological compartments at two distinct circadian time points representing peak (CT8) and trough (CT20) of circadian mortality rate upon LPS challenge. Cells from wild-type, genotype control and myeloid clock-knockout mice (n=5 per time point) were collected from a broad spectrum

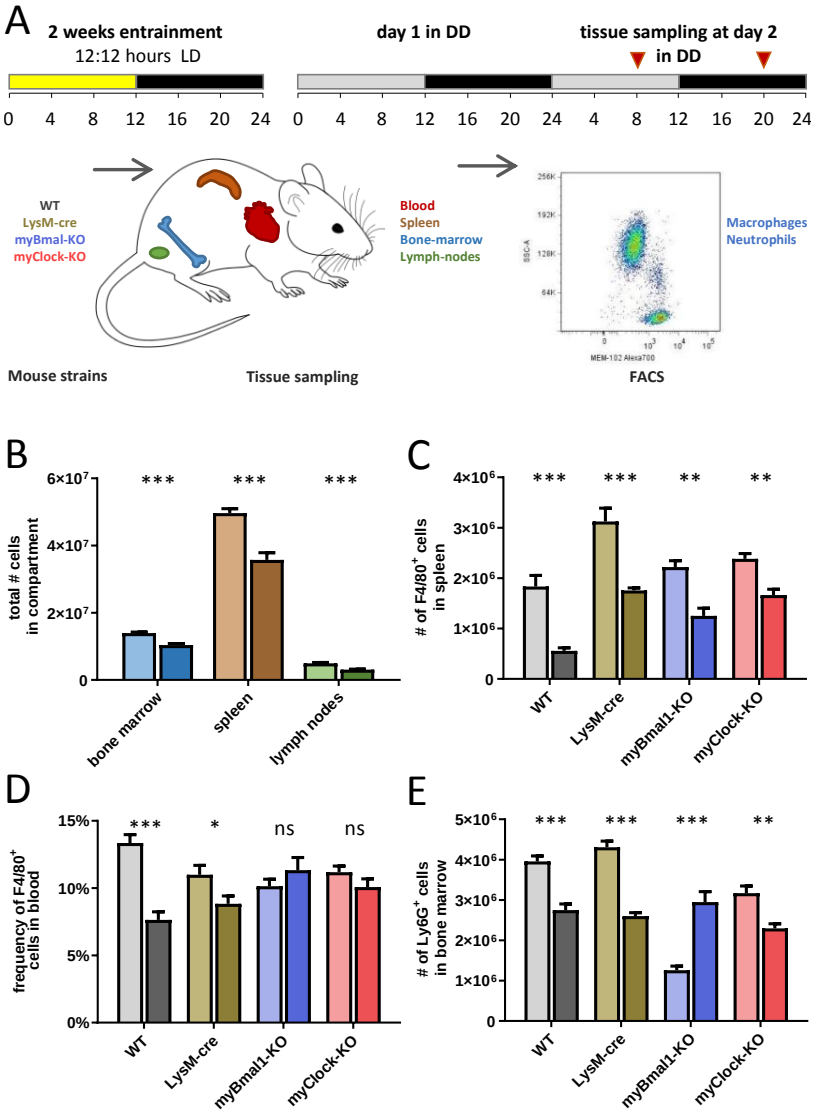

Figure 5. Circadian traffic of myeloid cells despite depletion of myeloid CLOCK or BMAL1. A) Experimental scheme to investigate time-of-day dependent immune cell traffic in various compartments and genetic mouse models. **B)** Total cell counts of femoral bone marrow (blue)), spleen (brown) or inguinal lymph nodes (green) at CT8 (light colors) or CT20 (dark colors). **C-E)** Cell number or frequency from wild-type and various conditional circadian clock mice at two different circadian time points (wild-type - gray, LysM-cre - brown, myBmal-KO - blue, myClock-KO - red, CT8 - light, CT20 - dark). **C)** Total number of F4/80$^+$ macrophages in spleen. **D)** Relative number of F4/80$^+$ macrophages in blood. Asterisks indicate level of significance as determined by t-test . **E)** Total number of Ly6G$^+$ neutrophils in bone marrow (significance levels: ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

of immune system compartments (blood, peritoneal cavity, bone marrow, spleen, inguinal lymph nodes, thymus)(Fig. 5*A*). As expected from previous studies [14,28,44] the number of cells were significantly time-of-day dependent in bone marrow, spleen and inguinal lymph nodes of wild-type mice (Fig. 5*B*).

If circadian traffic of myeloid cells and therefore lymphoid organ composition would be a main factor in regulating sensitivity to bacterial endotoxin, similar patterns in myeloid clock-knockout mice should be observed. However, results obtained from myBmal-KO and myClock-KO mice did not support this hypothesis. While in spleen, F4/80$^+$ macrophages of both myeloid clock knockout strains were still found at higher numbers at CT8 compared to CT20 (Fig. 5*C*), significant time-of-day differences of macrophage numbers in wild-type blood diminished in myeloid clock knockouts (Fig. 5*D*). Different patterns of time-of-day dependency were also observed in bone marrow (Fig. 5*E*), together suggesting a rather complex than mono-causal relation between myeloid clocks, circadian traffic and mortality risk in endotoxic shock.

Discussion

In this study we tested the hypothesis that local myeloid circadian clocks regulate the devastating immune response in endotoxic shock. A number of findings by various labs including our own pointed to such a possibility. First, monocytes/macrophages have been identified as important cellular entities relaying the endotoxin (LPS) triggered signal to the immune system and other organ systems by means of massive secretion of pro-inflammatory cytokines [40, 41]. Second, a high amplitude circadian clock in monocytes/macrophages has been shown to control circadian cytokine output *in vitro* and *ex vivo* [4,9,28]. Third, a high amplitude time-of-day dependent mortality has been demonstrated in endotoxic shock [23]. Fourth, a number of experiments done at two circadian time points, including a cecal ligation and puncture model [12], demonstrating loss of time-of-day dependency of mortality in myeloid *Bmal1* knockout mice [9] further supported this hypothesis.

On the other hand, some studies suggested other mechanisms, e.g. Marpegan and colleagues reported that mice challenged with LPS at two different time points in constant darkness did not exhibit differences in mortality rates [36], arguing for a light-driven process in regulating time-of-day dependency in endotoxic shock. However, we suspected that this result may be caused by a ceiling effect induced by constant darkness, since mortality rates at both time points were close to 100 percent. Indeed, when we compared susceptibility of mice challenged with a similar dose of LPS in light-dark versus constant darkness, we observed a marked increase of overall mortality. Similarly, depletion/mutation of *Per2* rendered mice insensitive to experimental time dependency in endotoxic shock [32]. Surprisingly, however, we found that depletion of either CLOCK or BMAL1 in myeloid lineage derived cells both did not abolish time-of-day dependency in mortality to endotoxic shock, which led us to reject our initial hypothesis.

Our data also exclude light as a stimulus driving mortality: First, data from Halberg, Marpegan, Scheiermann as well as our own lab [23, 28, 36, 44] suggest that mice are more susceptible to endotoxic shock during the light phase compared to dark phase - whereas switching light schedules from light-dark to constant darkness led to an increase in overall mortality. Second, irrespective of the genetic clock-gene depletion tested, our data unequivocally demonstrate circadian mortality rhythms upon LPS challenge even under DD conditions. Strikingly, peripheral blood cytokine levels showed - though altered - circadian time dependency in the myClock-KO strain.

A burst of cytokines, following a lethal dose of LPS is generally thought to be an indispensable factor causing multi-organ dysfunction and leading to death. However, the contribution of single cytokines has been difficult to tease apart due to complex nature of interconnected feedback systems. By adding time-of-day as an independent variable in a number of different mouse models we were able to correlate individual cytokines' contribution to mortality in the context of the complex response to LPS. While the pro-inflammatory cytokine TNFα was confirmed to act detrimentally, IL-18 surprisingly turned out to likely be protective. This interpretation is not only supported by the negative correlation of IL-18 levels in blood and mortality risk, but also by an anti-phasic oscillation of IL-18 levels paralleling those of the known protective cytokine IL-10.

While circadian regulation of trafficking lymphocytes by cell intrinsic clockworks have been demonstrated to impact the pathophysiology of an autoimmune disease model such as EAE [14], our data on distribution patterns of immune cells at two circadian time points draw a more complex picture. In spleen, absolute numbers of macrophages, but not neutrophils and monocytes, are independent of their local clocks. In contrast, patterns of macrophages and neutrophils in peripheral blood and bone marrow (respectively) change upon local myeloid clock depletion. Thus it appears that myeloid cell traffic is regulated at multiple levels including cell-intrinsic, endothelial and site specific factors. However, our data do not support the hypothesis of myeloid cell distribution being a main factor for time-of-day dependent mortality risk in endotoxic shock. Hence, what remains as the source of these rhythms?

Following the patho-physiology of endotoxic shock on its path from cause to effect, it is important to note that the bio-availability of bacterial endotoxin injected intraperitoneally depends on multiple factors (i.e. pharmaco-kinetics), many of which themselves might underlie circadian regulation. As a consequence, same doses of LPS administered *i.p.* at different times-of-day might result in highly diverging concentrations at the site of action.

Along this path, rhythmic feeding behavior driven by the central pacemaker was suggested to alter immune-responses directly or indirectly [29, 33]. Also, nutrition related factors could drive immune cells to respond differently to stimuli independent of local clocks [27, 39].

Finally, the ability of cells and organs to resist all sorts of noxa might as well be regulated in a time-of-day dependent manner. In this case, rather than same doses of toxin leading to time dependent cytokine responses (noxa) resulting in diverging rates of multi organ failure, same amount of noxa would cause time dependent rates of multi organ dysfunction and death. Indeed, work from Hrushesky [26] showed that mice challenged with same doses of TNFα at various times throughout the day exhibited time-dependent survival paralleling phenomena in endotoxic shock. However, our data showing fluctuating cytokine levels in myClock-KO mice challenged with LPS in constant darkness (Fig. 4A) question the mechanism of organ vulnerability as the only source of circadian regulation.

While our work suggests that local myeloid clocks do not account for time-of-day dependent mortality in endotoxic shock it unequivocally argues for a strong enhancing effect of myeloid CLOCK and BMAL1 on overall susceptibility, which adds on the effect of light conditions. However, it is important to note that our data do stay in conflict with findings from other labs which rather reported attenuating effects of BMAL1 on inflammation [9, 12, 39] but align well with a report on clock mutant mice [4]. Differences in genetic background of mouse strains, animal facility dependent microbiomes [42] or animal care procedures might account for this but remain unsatisfying explanations.

One of the most striking findings of our work is the large increase in susceptibility to endotoxic shock when mice were housed under DD as compared to LD conditions. This increase was observed in wild-type as well as in myBmal-KO mice, which implies that myeloid BMAL1 is not required for this effect. Interestingly, Carlson and Chiu reported similar effects in a cecal ligation and puncture model in rats upon transfer to LL (constant light) or DD conditions, where they found decreased survival as compared to rats remaining in LD conditions [6]. It is tempting to speculate that rhythmic light conditions, rather than light itself promote survival in endotoxemia. In either case, it will be important to further investigate these phenomena not only in respect to animal housing conditions, which need to be tightly light controlled in immunological, physiological and behavioral experiments but also for their apparent implications on health-care in intensive care units.

Materials and Methods

Animals

All procedures were authorized by and performed in accordance with the guidelines and regulations of the German animal protection law (Deutsches Tierschutzgesetz). Mice were housed in macrolon type II cages supplied with nesting material, food and water ad libitum at a 12h:12h light/dark (LD) cycle. For endotoxic shock and running wheel experiments mice were individually housed. For all other experiment mice were group-housed. Manipulations during the dark phase of the cycle were performed under infrared light. Male C57Bl/6 mice (Jackson Laboratories strain) mice were purchased from our animal facility (Charité FEM, Berlin, Germany) at 8-10 weeks of age. Homozygous, male *LysM*$^{Cre/Cre}$ (LysM-Cre) [8], myBmal-KO and myClock-KO were bred and raised in our animal facility (FEM, Berlin Germany) and used at 8-12 weeks. Female *LysM*$^{Cre/Cre}$ *Per2:Luc*, either wild-type or homozygous for *Clock*-flox, were bred and raised in our animal facility (FEM, Berlin Germany) and used at 14 weeks.

Generation of myeloid clock knockout mice

Bmal1$^{flox/flox}$ (Bmal-flox) [46] or *Clock*$^{flox/flox}$ (*Clock*-flox) [10] were bred with LysM-Cre to target *Bmal1* or *Clock* for deletion in the myeloid lineage. Offspring were genotyped to confirm the presence of the loxP sites within *Bmal1* or *Clock* and to determine presence of the Cre recombinase. Upon successful recombination the loxP flanked exon 8 of *Bmal1* or in case of *Clock* the floxed exon 5 and 6 were deleted. *LysM*$^{Cre/Cre}$ x *Clock*$^{flox/flox}$ were crossed onto a *Per2:Luc* [50] background for further characterization in bioluminescence reporter assays. All mice have been genotyped before experiments.

Endotoxic shock experiments

8-12 weeks old mice were entrained to 12h:12h light-dark cycles for 2 weeks. Dosing of LPS injection was adjusted for individual body weight prior injection. Injection volume did not exceed 10μl/g body weight. For LD experiments, mice were injected *i.p.* on day 14. For DD experiments mice were trans-

ferred to DD on day 14 and were injected *i.p.* on second day in DD. Animals were kept in the respective lighting conditions until the termination of experiment 60h post LPS injection. For all endotoxic shock experiments, human endpoints were applied to determine survival (for definition of human endpoints see respective section).

Intraperitoneal LPS injection

E. coli LPS (055.B5, Sigma Aldrich) stock solution (10mg/ml) was diluted to appropriate concentration in sterile PBS and thoroughly vortexed before use. LPS injection was performed *i.p.* using Legato 100 Syringe Pump (KD Scientific). The following settings were applied: Mode: infuse only; syringe: BD, plastic, 5ml; rate: 2ml/min. A cannula (26G x 3/8") was attached to Legato 100 by microbore extension line (60cm, MedEx, Smiths Medical) for the LPS injections.

Time-of-day dependent mortality experiments

Endotoxic shock mortality experiments were comprised of two parts for each mouse strain and condition tested: First, lethal dose 50 (LD_{50}) of LPS was determined by injecting groups of mice (n=10) at four 6-hour spaced time points. The LD_{50} describes the concentration at which approx. 50% of all animals injected (averaged over all injection time points) survive. This ensures most dynamic range for the detection of potential circadian rhythms. Second, experimentally determined LD_{50} of LPS was used to investigate, whether mortality by endotoxic shock was dependent on time-of-day. To this end, mice (n=14 per group) were injected *i.p.* at six 4-hour spaced time points to increase statistical power for circadian rhythm analysis (see statistical data analysis section).

Definition of human endpoints

A scoring system was developed in order to detect irreversibly moribund mice before the occurrence of death by endotoxic shock. It is based on previous reports by [32, 45] and required further refinements according to our experience. Mice in the endotoxic shock experiments were monitored and scored every 2-4 hours for up to 60 hours post LPS injection. In addition, surface body temperature was measured at the sternum every 12h and body weight was measured every 24 h. Mice with a score of 0-2 were monitored every 4h. As of a score of 3, the monitoring frequency was increased to every 2h. In addition, softened, moisturized food was provided in each cage as of a score of 3. Weight loss exceeding 20%, 3 consecutive scores of 4, or one score of 5 served as human endpoints. A mouse was defined as a non-survivor when human endpoints applied and was subsquently sacrificed by cervical dislocation. Mice which did not display any signs of endotoxic shock such as weight or temperature loss and no increasing severeness in score were excluded from the analysis.

Peripheral blood cytokine concentrations in endotoxic shock

To determine the blood cytokine levels in the endotoxic shock model, mice were injected with corresponding LD_{50} of LPS at six 4-hour spaced time points (n=14). 2h post LPS injection mice were terminally bled by cardiac puncture using a 23G x 1" cannula (Henke-Sass Wolf). Syringes (1ml, Braun) were coated with heparin (Ratiopharm) to avoid blood coagulation. After isolation blood was kept at 4°C for subsequent plasma preparation. To this end, blood was centrifugated for 15min, 370g at 4°C. Plasma was isolated, aliquoted and frozen at -80°C for further analysis.

Isolation of peritoneal macrophages (PM)

Mice were sacrificed by cervical dislocation. Peritoneal cavity cells (PEC) were isolated by peritoneal lavage with ice cold PBS. Lavage fluid visibly containing red blood cells was dismissed. For RNA measurements or bioluminescence recordings peritoneal macrophages were further purified by MACS sorting (Miltenyi) according to manufacturer's protocol using mouse/human CD11b Microbeads and LS columns. Eluates containg positively sorted macrophages were analyzed for sorting efficiency by FACS. All steps were performed at 4°C.

Isolation of bone marrow cells

Mice were sacrificed by cervical dislocation. One tibia and femur were excised per mouse. Femur and tibia were flushed with supplemented RPMI 1640 and erythrocytes were lysed using GEYS solution (2min at 4°C). After erythrocyte lysis, cells were suspended in supplemented RPMI 1640 medium and filtered through a 30μM filter (Miltenyi). Cell numbers were determined using a Neubauer chamber. All steps were performed at 4°C. All centrifugation steps were performed at 4°C, 300g, 7min.

Isolation of spleen cells

Mice were sacrificed by cervical dislocation. Spleen was removed and a single cell single cell suspension was obtained using gentleMACS, Miltenyi (program: m_spleen_01) with C-tubes (Miltenyi) in PBS. Next single cell solution was filtered using 100μM cell strainers (Thermo Fischer). GEYS solution was used

for erythrocyte lysis for 2min at 4°C. After erythrocyte lysis cells were suspended in supplemented RPMI 1640 and filtered through a 30μM filter (Miltenyi). Cell number was determined using a Neubauer chamber. All steps were performed at 4°C. All centrifugation steps were performed at 4°C, 300g, 7min.

Bioluminescence recordings

PER::LUC protein bioluminescence recordings were used to characterize circadian clock function in peritoneal macrophages, SCN and lung tissue. Mice were sacrificed by cervical dislocation. Brains and lungs were isolated and transferred to chilled Hank's buffered saline solution, pH 7.2. (HBSS). For tissue culture, 300μm coronal sections of the brain and 500μm sections of the lung were obtained using a tissue chopper. The lung and SCN slices were cultured individually on a Millicell membrane (Millipore) in a Petri dish in supplemented DMEM containing 1μM luciferin (Promega). PECs were isolated by peritoneal lavage and immediately CD11b-MACS sorted. The CD11b-sorted peritoneal macrophages were cultured in Petri dishes in supplemented DMEM containing 1μM luciferin (Promega). For bioluminescence recording, tissues/primary cell cultures were placed in light-tight boxes (Technische Werkstaetten Charite, Berlin, Germany) equipped with photo-multiplier tubes (Hamamatsu, Japan) at standard cell culture conditions. Bioluminescence was recorded in 5min bins. On fifth day in culture PMs were treated with 1μM dexamethason for 1h, followed by a medium change to supplemented DMEM containing 1μM luciferin (Promega). Data were further processed and analyzed using Chronostar 3.0 [34].

Generation of whole cell protein lysates

Liver and peritoneal cavity cells were homogenized in ice cold RIPA buffer containing 1x protease-inhibitor-cocktail (Sigma Aldrich) and incubated on ice for 30min. Homogenized cells were then centrifuged at maximum speed for 30min at 4°C to pellet the insoluble cell debris. The supernatant fraction was then removed and used for cellular protein analysis or frozen at -80°C. Protein concentrations were determined using standard BCA assay.

Immunoblotting

Samples were denaturated for SDS-PAGE in NuPAGE SDS Sample Buffer (4x) (Invitrogen) containing 0.8% 2-β-mercaptoethanol (Sigma) and boiled for 5-10min at 95°C. SDS-PAGE using 4-12% Bis-Tris gels (Thermo Scientific) at 200V for 60min in NuPAGE MES SDS Running Buffer. Proteins were transferred to a nitrocellulose membrane (0.45μm) using a tank transfer system (wet transfer). NuPAGE transfer buffer, containing 20% Methanol, was cooled with an ice block to prevent overheating during the transfer. The transfer was run for 120min at 90V. Following the transfer, the membrane was blocked in TBS-T with 5% non-fat, dry milk for 1-2h at RT. After a washing step in TBS-T (3 x 10min), the membrane was placed in the primary antibody solution (TBS-T with 5% non-fat, dry milk) and gently shaken overnight at 4°C. The membrane was then washed in TBS-T (3 x 10min) and incubated with the HRP-conjugated secondary antibody (Santa Cruz Biotechnologies) in TBST-T for 2h at RT. After another washing step in TBS-T (3 x 10min), a chemiluminescence reaction was performed with Super SignalWest Pico substrate (Pierce). The protein bands were visualized using the ChemoCam detection system (Intas). The following primary antibodies were used: murine CLOCK - rabbit anti-mCLock (Bethyl Laboratories, A302-618A) , murine BMAL1 - rabbit anti-mBMAL1 (kind gift from Micheal Brunner) , murine ACTINB - mouse anti mBactin (Sigma, A5441). Secondary antibodies used: goat anti-mIgG-HRP (SantaCrz Biotechnology, sc-2005), donkey anti-rbIgG-HRP (SantaCrz Biotechnology, sc-2005).

Single- and multiplex immunoassays

Singleplex assay: murine IL-6 plasma concentration was determined by ELISA according to manufacturer's protocol (Ebioscience) in a 96-well format (Corning). Plasma samples were diluted 1:200 in supplied assay buffer. Absorption was measured at 470nm. Reference wavelength was measured at 560nm by Infinite F200Pro plate reader (Tecan). Multiplex assay: 13 cytokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL7/MCP-3, CXCL5, IL-1α, IL-1β, IL-10, IL-12p40, IL-18, Eotaxin, Rantes/CCL5, TNFα) were assessed using the ProCartaPlex, Mix and Match, Mouse 13-Plex (Affymetrix, eBioscience). ProCartaPlex was performed as described in manufacturer's protocol in a 96 well format (eBioscience). All washing steps were performed using a hand held magnetic washer (eBioscience). Data were acquired using a MagPix (Luminex) detection device. Data evaluation was performed using ProcartaPlex Analyst v.1.0 (eBiosciences).

Isolation and quantification of RNA

Total RNA was isolated using the PureLink RNA Mini Kit (Ambion) according to the manufacturer's manual. In addition, an on-column DNA digestion was performed using PureLink DNase Set (Life Technologies). RNA was quantified by measuring the absorption at 260nm with NanoDrop 2000C (Thermo Scientific).

Quantitative real-time PCR

Total RNA was reverse-transcribed to cDNA using random hexamers to prime reverse transcriptase reaction. cDNA was diluted 1:10 in H_2O for use in qRT-PCR. qRT-PCR was performed using a 2-step protocol with the following primer-sets: primer-sets for mCry1, mCry2, mDpb, mNr1d1, mNpas2, mPer1, mPer2 were purchased from Qiagen (QT00117012, QT00168868, QT00103089, QT00164556, QT00108647, QT00113337, QT00198366, respectively). mGapdh, fwd: ACGGGAAGCTCACTGGCATGGCCTT, rev: CATGAGGTCCACCACCCTGTTGCTG; mBmal1 primers were designed to characterize myBmal-KO mice. Forward primer (fwd: GGACACAGACAAAGATGACCC) binds upstream of exon 8 and the reverse primer (rev: TTTTGTCCCGACGCCTCTTT) within exon 8 of *Bmal1*. Thus after successful Cre recombination, exon 8 is deleted and no PCR product is detectable. *Clock* primers were designed to characterize myClock-KO mice. Primers bind in exon 5 (fwd: ATTGGTGGAAGAAGATGACAAGGA) and in exon 6 (rev: TACCAGGAAGCATAGACCCC) of clock. As exon 5 and 6 are flanked by loxP sites, after successful Cre recombination no PCR product is amplified.

Flow cytometry (FACS)

Two panels of antibodies were established to target a broad range of immune cells in various sites of the organism. Before each experiment, antibody mix of both panels were prepared and kept on 4°C for labeling of all samples of the respective experiment in order to minimize intra-experimental variability. All antibody-mixes were prepared in FACS buffer containing 1:50 FcR blocking reagent. FACS staining of samples: 100μL of the cell suspensions were transferred into a 96-well plate and spun down for 7min, 300g at 4°C. Supernatant was carefully discarded and pellet re-suspended in 50μL of mastermix containing one of two antibody panels. Cells were incubated for at least 30mins at 4°C in darkness. Subsequently 200μL FACS buffer was added and cells were centrifuged for 7min, 300g at 4°C. Cells were washed twice using 200μL FACS buffer each before fixation in 200μL 4% PFA for 30min at RT. Finally, cells were spun down and re-suspended in 200μL FACS buffer and stored at 4°C for up to a week before FACS data acquisition in a FACS CantoII (BD Biosciences).

Statistical data analysis

Statistical analysis was performed in GraphPad Prism 8 and R. Normality was tested using Shapiro-Wilk normality test. When data were normally distributed and two parameters were compared, One-way ANOVA with Dunnett's multiple comparison as a post hoc test was applied. When comparing more than two groups and two parameters a two-way ANOVA with Bonferonni's post hoc test was applied. Two sample comparison was performed with 2-sided students t-test for normally distributed data and Mann-Whitney U test for non-normally distributed data. Time-of-day dependent mortality experiments: Mortality data were transformed to probability of death (between 0-1) in order to compute sine fit using alogistic regression. The confidence interval was derived from sine fit estimation. After statistical analysis, the mortality data was transformed back to the initial percentages. Cross-correlation analysis of morality and cytokine data: To correlate mortality rates and cytokine levels across all animal models, a permutation of sine fit of the mortality data and plasma cytokine data was used. This bootstrapping (randomization) procedure gives rise to the empirical distribution of correlations. The p-value is the fraction of randomizations that gave a correlation with the opposite denominator, meaning that a p=0.05 means that only 5% of correlations crossed zero correlation threshold. For linear regression analysis of the summary of cytokine and mortality data, the estimated mortality rate determined by sine fit of mortality data was correlated to mean value of plasma cytokine concentration using Spearman's rank correlation. Bioluminescence recordings were analysed using in-house written software ChronoStar 3.0 [34]. In brief, raw bioluminescence counts were transformed to log-space and trends removed by subtracting the 24h running average. Circadian rhythm parameters were estimated by fitting a damped sine wave to these data. Finally, data were reversely transformed into linear space. Circadian rhythmicity of cytokine time-series was tested using our in-house written software ChronoLyse. In brief, a 24h sine wave was fitted the beforehand log-transformed data and parameters of fit were used to estimate amplitude, phase and mean levels. Rhythmicity was tested by testing against a flat line using F-test.

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