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Circadian rhythms of macrophages are altered by the acidic pH of the tumor microenvironment.

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Abstract

Macrophages are prime therapeutic targets due to their pro-tumorigenic and immunosuppressive functions in tumors, but varying efficacy of therapeutic approaches targeting macrophages highlights our incomplete understanding of how the tumor microenvironment (TME) can influence regulation of macrophages. The circadian clock is a key internal regulator of macrophage function, but how circadian rhythms of macrophages may be influenced by the tumor microenvironment remains unknown. We found that conditions associated with the TME such as polarizing stimuli, acidic pH, and elevated lactate concentrations can each alter circadian rhythms in macrophages. Circadian rhythms were enhanced in pro-resolution macrophages but suppressed in pro-inflammatory macrophages, while acidic pH had divergent effects on circadian rhythms depending on macrophage phenotype. While cyclic AMP (cAMP) has been reported to play a role in macrophage response to acidic pH, our results indicate that pH-driven changes in circadian rhythms are not mediated solely by the cAMP signaling pathway. Remarkably, clock

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correlation distance analysis of tumor-associated macrophages (TAMs) revealed evidence of circadian disorder in TAMs. This is the first report providing evidence that circadian rhythms of macrophages are altered within the TME. Our data suggest that heterogeneity in circadian rhythms at the population level may underlie this circadian disorder. Finally, we sought to determine how circadian regulation of macrophages impacts tumorigenesis, and found that tumor growth was suppressed when macrophages had a functional circadian clock. Our work demonstrates a novel mechanism by which the tumor microenvironment can influence macrophage biology through altering circadian rhythms, and the contribution of circadian rhythms in macrophages to suppressing tumor growth.

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Introduction

Tumor-associated macrophages (TAMs) are one of the most abundant leukocytes found in solid tumors, with high intra-tumoral TAM density generally associated with poor clinical outcome [1-3]. This is consistent with the largely pro-tumorigenic role of macrophages within tumors[4]. Macrophages are highly plastic professional phagocytes whose ability to sense and respond to the environment makes them uniquely equipped to protect tissue integrity under normal homeostatic conditions[5, 6]. However, within the chronically inflamed tumor microenvironment (TME), failure to resolve the inflammation can lead to uncontrolled secretion of tissue repair factors by TAMs, promoting tumor growth and metastatic capacity[7-9]. At the same time, conditions in the TME can drive TAMs to suppress potentially anti-tumorigenic inflammatory activity through various mechanisms including secretion of anti-inflammatory cytokines and expression of checkpoint inhibitors such as PD-L1, promoting immune suppression[4, 10-16].

TAMs are known to suppress the response to many standard of care treatments through their pro-tumorigenic and immunosuppressive functions, making them a prime therapeutic target[17]. However, we still have an incomplete understanding of how the TME influences macrophages, limiting our ability to target them; this is highlighted by the varying efficacy of therapeutic approaches used to target macrophages[18]. This is thought to be due in part to the significant phenotypical heterogeneity of TAMs within tumors[18-20]. Evidence suggests this heterogeneity is due to the ability of macrophages to sense and adapt to the local microenvironment, which varies within tumors depending on several factors including the distance from blood vessels and neighboring cells[21-24]. Indeed, various conditions in the TME have been shown to influence macrophage phenotype and function[4].

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In particular, poor vascularization of solid tumors leads to inefficient delivery of oxygen, creating regions of hypoxia[25]. The hypoxic response promotes enhanced glycolytic activity of cells within the region, which, coupled with poor tissue drainage as a result of leaky vasculature, results in elevated levels of protons and lactate, acidifying the microenvironment[26, 27]. Of conditions in the TME, it has been well appreciated that acidic pH can promote a pro-resolution phenotype, thereby contributing to the pro-tumorigenic and immunosuppressive functions of macrophages within tumors[28-30].

The myriad ways in which the TME can impact regulation of macrophages remain to be fully elucidated. Circadian rhythms are a key regulatory system that is present in almost all cells of the body, and are an understudied facet of macrophage biology[31]. Acidic pH is a condition commonly associated with the TME that has been shown to alter circadian rhythms in cell lines[32]; however, whether pH influences circadian rhythms in macrophages remain unknown.

Circadian rhythms are 24-hour rhythms that impart oscillations in the levels of circadian-regulated gene transcripts and proteins in a tissue- and cell-specific manner, resulting in time-of-day-dependent variation in many cellular processes[33, 34]. The molecular clock, which we will refer to as the circadian clock, drives these rhythms in cells through a cell-autonomous transcription/translation feedback loop, which is controlled in part by the transcription factor BMAL1[31]. Circadian clocks are synchronized by signals sent out from the central circadian clock housed within the suprachiasmatic nucleus of the hypothalamus, which entrains circadian clocks to the time of day[31]. This allows for the temporal coordination of cells in spatially distinct tissues, although how the local microenvironment influences circadian rhythms remains to be elucidated.

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All leukocytes tested to date have functional circadian clocks[35-42]. As such, nearly every arm of the immune response (both innate and adaptive) is subject to circadian regulation[35, 37, 43]. Time-of-day-dependent regulation of immune responses is achieved though temporal gating of response to stimuli, effector function, and cell trafficking[42, 44-48], all of which promote coordination between the multiple phases of the immune response[40, 42, 49-53].

Key aspects of macrophage function are subject to circadian regulation, including cytokine secretion and phagocytosis[36, 44, 45, 51, 54]. This results in a time-of-day-dependent macrophage response to stimuli, which modulates the magnitude of the resulting adaptive immune response and determines disease progression[44, 51, 55]. Circadian regulation of macrophages is of particular interest given recent evidence of time-of-day variation in the frequency of TAMs expressing surface markers associated with phenotype[56-58]. A promising application of such circadian variation was made evident in leveraging observations of circadian frequency in TAMs expressing immune checkpoint blockade (ICB) target PD-1 to increase efficacy of PD-1/PD-L1 ICB therapy by timing treatment to the time of day when PD-1+ TAMs were most frequent[57, 59]. This suggests that leveraging time-of-day variations in therapeutic targets could be promising avenue to increase efficacy, highlighting the importance of understanding how circadian rhythms of macrophages may be influenced by conditions in the TME, which remains unclear.

In this work, we present evidence that circadian rhythms of macrophages are altered in the TME. We uncover a novel way in which two conditions within the TME, acidic pH and lactate, can influence macrophage biology through modulation of circadian rhythms. We also find that macrophages of different phenotypes have distinct circadian rhythms. Remarkably, we found evidence of circadian disorder in tumor-associated macrophages, indicating that circadian rhythms are altered in macrophages

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within the TME. Furthermore, our data suggest that heterogeneity in circadian rhythms at the population level may underlie the observed circadian disorder. This work elucidates a novel way in which the TME can alter macrophage biology, and represents the first steps to understanding how the tumor microenvironment can alter circadian rhythms of immune cells such as macrophages.

Results

Macrophages of different phenotypes exhibit different circadian rhythms.

As macrophages are a phenotypically heterogeneous population in the TME, we first sought to understand whether diversity in macrophage phenotype could translate to diversity in circadian rhythms of macrophages. To this end, we used the well-established *in vitro* polarization models of IL-4 and IL-13-stimulated (pro-resolution, often referred to as 'M2' and used as a model of pro-tumorigenic macrophages) and IFNγ and LPS-stimulated (pro-inflammatory, often referred to as 'M1' and broadly viewed as anti-tumorigenic) macrophages. Arginase 1 (ARG1; *Arg1*) and Inducible Nitric Oxide Synthase (iNOS; *Nos2*) are upregulated in pro-resolution and pro-inflammatory macrophages, respectively, and thus are commonly used as markers of the pro-resolution or pro-inflammatory phenotype in macrophages[60]. Consistent with previous studies, we found that *Arg1* was induced in IL-4 and IL-13-stimulated macrophages, but not IFNγ and LPS-stimulated macrophages; in contrast, *Nos2* was induced in IFNγ and LPS-stimulated macrophages, but not IL-4 and IL-13-stimulated macrophages (Supplementary Figure 1). This indicates that macrophages stimulated with IL-4 and IL-13 were polarized toward a pro-resolution phenotype, while macrophages stimulated with IFNγ and LPS were polarized toward a pro-inflammatory phenotype.

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Circadian rhythms of macrophages were measured by monitoring PER2, a key component of the circadian clock, via the rhythmic activity of the PER2-Luciferase (PER2-Luc) fusion protein in a live cell LumiCycle luminometer (Supplementary Figure 2A)[61]. Bone marrow-derived macrophages (BMDMs) were generated from bone marrow of mice expressing PER2-Luc. Following differentiation, the circadian clocks of BMDMs were synchronized, and rhythms were observed for up to 4 days (Figure 2B).

To determine whether phenotype can influence circadian rhythms in macrophages, BMDMs were cultured in the presence or absence of polarizing stimuli, and rhythms were observed by LumiCycle (Figure 1). The amplitude of rhythms is the magnitude of change between the peak and the trough, and is indicative of the strength of rhythms[62, 63]. Amplitude of rhythms was suppressed in pro-inflammatory macrophages compared to unstimulated macrophages. In contrast, amplitude of rhythms in pro-resolution macrophages was enhanced. This suggests that rhythms are suppressed in pro-inflammatory macrophages but enhanced in pro-resolution macrophages, which agrees with previous observations[64, 65]. Period is the amount of time it takes to complete one full oscillation, and is indicative of how fast the circadian clock is running[66]. Compared to unstimulated macrophages, period was lengthened in pro-resolution macrophages but shortened in pro-inflammatory macrophages. This In line with others' observations, and suggests that the clock runs slower in pro-resolution macrophages but runs faster in pro-inflammatory macrophages[64, 65].

Interestingly, we observed differences in damping of rhythms in polarized macrophages. Damping is measured as the number of days required for the amplitude of rhythms to decrease by 30% of the first cycle[67]. Damping of rhythms in most free-running cell populations, cultured in the absence of external synchronizing stimuli, occurs naturally as the circadian clocks of individual cells in the population become desynchronized from each other; thus, damping can be indicative of desynchrony within a

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156 population[68]. The damping rate increases as the number of days required for rhythms to damp
157 decreases; conversely, as damping rate decreases as the number of days required for rhythms to damp
158 increases. We observed increased rate of damping in pro-inflammatory macrophages compared to
159 unstimulated macrophages, indicating that population-level rhythms were maintained for a shorter
160 period of time in pro-inflammatory macrophages. In contrast, damping rate was decreased in pro-
161 resolution macrophages, indicating that population-level rhythms were maintained for longer in pro-
162 resolution macrophages. These data suggest that pro-inflammatory macrophages may have an impaired
163 ability to maintain synchrony, while pro-resolution macrophages may have an enhanced ability to
164 maintain synchrony.
165
166 Collectively, these data suggest that pro-inflammatory macrophages have weaker rhythms and impaired
167 ability to maintain synchrony, while pro-resolution macrophages have enhanced rhythms and an
168 increased ability to maintain synchrony. This is evidence that macrophages of different phenotypes have
169 distinct circadian rhythms, suggesting that diversity of macrophage phenotype may lead to diversity in
170 macrophage circadian rhythms.
171
172 **Acidic pH alters circadian rhythms of macrophages.**
173
174 The TME has previously been shown to be acidic, with a pH ranging from 6.8 to 6.3; much more acidic
175 than the typical pH in blood and healthy tissue of 7.3-7.4[69-71]. There has been a previous report in
176 which acidic pH can alter circadian rhythms, but whether this applies to macrophages remains
177 unknown[32]. Thus, we cultured BMDMs under conditions of varying pH within a range that mimics that
178 found within the TME (pH7.4-pH6.5). As macrophages are a heterogeneous population in the TME, we
179 assessed the influence of acidic pH on rhythms of unstimulated, pro-resolution, and pro-inflammatory

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macrophages. In line with previous observations, macrophages cultured at pH 6.5 were polarized toward a pro-resolution phenotype, characterized by increased expression of *Arg1* and *Vegf* compared to macrophages cultured at pH 7.4 (Supplementary Figure 3A). Pro-inflammatory macrophages cultured at pH 6.5 had decreased expression of *Nos2* (iNOS) compared to those cultured at pH 7.4, suggesting that an acidic pH of 6.5 both promotes a pro-resolution phenotype and suppresses a pro-inflammatory phenotype.

It has been observed that inducible cyclic AMP early repressor (*Icer*), an isoform of cyclic AMP (cAMP)-response modulator (*Crem*), is upregulated downstream of acid-sensing in macrophages, and has been used as a "biomarker" for macrophages exposed to acidic conditions in tumors. We observed induction of *Icer* in unstimulated and pro-resolution macrophages cultured at pH 6.5 compared to pH 7.4, indicating that these macrophages were sensing acidic conditions (Supplementary Figure 3B). In line with previous observations that *Icer* is induced downstream of LPS-driven TLR4 signaling, *Icer* was upregulated in pro-inflammatory macrophages compared to unstimulated macrophages even at neutral pH 7.4[72]. Although *Icer* was not further upregulated in pro-inflammatory macrophages at pH 6.5 compared to pH 7.4, *Nos2* was suppressed at pH 6.5 compared to pH 7.4, suggesting that pro-inflammatory macrophages responded to acidic pH. In all, these data confirm that macrophages of various phenotypes can sense and respond to acidic conditions within the range of pH found in the TME.

To determine whether an acidic microenvironment can influence circadian rhythms in macrophages, we assessed rhythms of unstimulated, pro-resolution, and pro-inflammatory macrophages under normal and acidic conditions. To this end, BMDMs were polarized toward a pro-resolution or a pro-inflammatory phenotype, or left unstimulated, and cultured in media at a normal pH of 7.4 or at acidic pH of 6.8 or 6.5; PER2-Luc rhythms were then observed by LumiCycle. In unstimulated and pro-resolution BMDMs, lower

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pH led to enhanced amplitude, a shortening in period, and increased damping rate of rhythms at pH 6.8 and pH 6.5 relative to neutral pH 7.4 (Figure 2A,B). This suggests that in unstimulated and pro-resolution macrophages, acidic pH can strengthen rhythms by enhancing amplitude and speed up the circadian clock, but may impair ability to maintain synchrony. Notably, changes in amplitude and period occurred in a dose-dependent fashion as pH decreased, indicating that rhythms are altered in a pH-dependent manner. In contrast, pro-inflammatory macrophages cultured at pH 6.5 exhibited suppressed amplitude, elongated period, and decreased damping rate of rhythms compared to those cultured at pH 7.4 (Figure 2C). This suggests that in pro-inflammatory macrophages, acidic pH can weaken rhythms by decreasing amplitude and slow down the speed of the clock, but may promote the ability to maintain synchrony. Low pH was also observed to alter the expression of the circadian clock genes *Per2*, *Cry1*, and *Nr1d1* (REV-ERBα) over time across different macrophage phenotypes (Figure 2D,E,F). These data indicate that exposure to acidic pH can induce changes in circadian rhythms of macrophages. Interestingly, while rhythms of unstimulated and pro-resolution macrophages are enhanced under acidic pH despite increased damping rate, rhythms of pro-inflammatory macrophages are suppressed under acidic conditions but have improved damping rate. This suggests that acidic pH modulates rhythms differently in macrophage of different phenotypes. The observation that acidic pH can enhance rhythms is particularly interesting, given that acidic pH is a stressful condition that can compromise macrophage survival (Supplementary Figure 4)[30].

While BMDMs are a widely used model for studying macrophages *in vitro*, there are biological differences between BMDMs generated in culture and tissue-resident macrophages differentiated *in vivo*. Thus, we sought to determine whether our observations of pH-induced changes in rhythms were relevant to macrophages differentiated *in vivo*. To this end, we harvested peritoneal macrophages from mice expressing PER2-Luc in the morning at ZT0 (6 AM) or in the evening at ZT12 (6 PM). Peritoneal

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macrophages were cultured in media at neutral pH of 7.4 or acidic pH of 6.5 and observed by LumiCycle. Recapitulating our results in BMDMs, peritoneal macrophages exhibited increased amplitude, decreased period, and increase rate of damping at pH 6.5 compared to pH 7.4 (Figure 3A). These data indicate that pH-dependent changes in circadian rhythms are relevant to *in vivo*-differentiated macrophages.

Circadian rhythms confer time-of-day variability in response to stimuli. As we have observed that acidic pH can influence circadian rhythms of macrophages, we sought to understand if macrophages may be more or less susceptible to pH-induced changes in rhythms depending on time of day of exposure. To this end, we compared the magnitude of change in amplitude, period, and damping in peritoneal macrophages when exposed to acidic pH 6.5 compared to neutral pH 7.4 at different times of day (Figure 3B). We observed no significant difference in the pH-driven change in amplitude, period, or damping in rhythms of peritoneal macrophages taken in the morning at ZT0 compared to those taken in the evening at ZT12. This indicates that the influence of pH on rhythms of macrophages was similar when exposed to acidic pH in the morning or in the evening, which suggests that macrophages are similarly susceptible to pH-induced changes in rhythms regardless of time of day of exposure.

Lactate alters circadian rhythms of macrophages in a manner distinct from acidic pH.

Elevated lactate concentrations often co-localize to regions of high acidity, due to the export of both protons and lactate by glycolytic cells[73-75]. In tumors, concentration of lactate has been observed to be present in concentrations of 30mM, elevated over typical lactate levels in blood and healthy tissue of 1.5-3mM[76]. There are previous reports that lactic acid can promote polarization of macrophages toward a pro-resolution phenotype[77]. Thus, we sought to understand if lactate may be a feature of the TME capable of influencing circadian rhythms of macrophages in addition to acidic pH. To this end, we

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252 cultured BMDMs in the presence or absence of 25 mM sodium-L-lactate. In line with previous
253 observations, BMDMs exposed to lactate had elevated levels of *Vegf*; however, we did not observe
254 significant elevation of *Arg1* (Figure 4A)[77].
255
256 We then cultured BMDMs at normal pH 7.4 or acidic pH 6.5, in the presence or absence of 25 mM
257 sodium-L-lactate, and monitored circadian rhythms (Figure 4). Rhythms of BMDMs at pH 7.4 exposed to
258 25 mM sodium-L-lactate had elongated period and decreased damping time compared to BMDMs
259 cultured at pH 7.4 without sodium-L-lactate (Figure 4B). This suggests that lactate can slow down the
260 circadian clock and may impair the ability of macrophages to maintain synchrony. Interestingly, these
261 changes in rhythms are different from those observed in under acidic conditions, indicating that lactate
262 can modulate circadian rhythms in macrophages in a manner distinct from acidic pH.
263
264 As previously observed, macrophages exposed to acidic pH 6.5 exhibited increased amplitude, shortened
265 period, and increased damping rate of circadian rhythms. When BMDMs were exposed to both acidic pH
266 and elevated lactate, the increased amplitude observed at pH 6.5 is maintained; however, the shortened
267 period observed at pH 6.5 is lost, with period lengthened in BMDMs cultured in 25 mM sodium-l-lactate.
268 The increased damping rate of rhythms in BMDMs cultured at pH 6.5 compared to pH 7.4 is maintained,
269 and is further dampened by exposure to 25 mM sodium-l-lactate. These data indicate that changes in
270 rhythms associated with acidic conditions persisted when co-exposed to elevated lactate. In all, these
271 data indicate that concentration of lactate similar to that present in the TME can influence circadian
272 rhythms of macrophages. Lactate altered rhythms differently than acidic pH, and when macrophages
273 were exposed to acidic pH and lactate together, rhythms were further altered. This suggests that when
274 macrophages are exposed to multiple conditions capable of altering circadian rhythms, each condition
275 may contribute to a combined effect on rhythms that differs from its individual impact.

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Induction of cAMP signaling alone is not sufficient to fully drive changes in circadian rhythms associated with acidic pH.

Evidence in the literature suggests that acidic pH is primarily sensed by macrophages via certain G protein-coupled receptors (GPCRs), inducing an increase in intracellular cAMP that drives downstream signaling through the cAMP pathway[29]. Transcriptional changes downstream of cAMP signaling subsequently promotes a pro-resolution phenotype[29, 78, 79]. Downstream of cAMP signaling, transcription of the *Crem* isoform *Icer* is induced, which has been used as a "biomarker" for macrophages exposed to acidic conditions in tumors[29]. In line with previous reports, we have observed induction of *Icer* in macrophages under acidic pH, suggesting that cAMP signaling is being induced under acidic conditions (Supplementary Figure 2A)[29]. This occurs as early as 2 hours, concurrent with changes in rhythms, which are observed by 6 hours following exposure to acidic conditions. It has been shown that induction of cAMP signaling alone is sufficient to drive a pro-resolution phenotype in macrophages similar to that observed under acidic conditions[29, 78]. Additionally, cAMP signaling has been previously observed to modulate circadian rhythms in SCN and rat fibroblasts[80, 81]. Thus, we sought to understand if the cAMP signaling pathway may be mediating the pH-induced changes in circadian rhythms in macrophages.

We first sought to determine if elevation in intracellular cAMP alone was sufficient to drive changes in rhythms observed in macrophages under acidic conditions. To this end, we treated macrophages with forskolin, an adenylyl cyclase activator that stimulates production of cAMP, or IBMX, which drives accumulation of cAMP through inhibition of phosphodiesterases (PDEs). We used a range of doses similar to those previously shown to induce cAMP signaling in macrophages in the literature[29, 80, 82].

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Treatment with either forskolin or IBMX increased amplitude of rhythms in macrophages, but not to the same magnitude as acidic pH, and did not result in a changed period (Figure 5). Moreover, amplitude of rhythms was not altered in forskolin- or IBMX-treated macrophages at pH 6.5, indicating neither forskolin treatment nor IBMX treatment had any additional effect on rhythms under acidic conditions. These data indicate that in macrophages, cAMP signaling alone induces enhanced amplitude of rhythms similar to low pH, but the magnitude of this change is far less; additionally, period, which is altered under acidic conditions, remains unchanged. This suggests that cAMP signaling alone may contribute to but is not sufficient to fully recapitulate the changes in rhythms observed under acidic conditions.

Adenylyl cyclase inhibitor MDL-12330A can suppress pH-mediated changes in amplitude of circadian rhythms and pro-resolution phenotype without suppressing cAMP signaling.

To further test whether pH-induced changes in rhythms are mediated by cAMP signaling, we treated BMDMs with MDL-12330A (henceforth referred to as MDL-12), an adenylyl cyclase inhibitor which has previously been shown to suppress cAMP signaling in macrophages under acidic conditions[29]. As previously observed, amplitude of rhythms was enhanced in macrophages cultured at pH 6.5, while period was shortened and damping rate of rhythms was increased. When BMDMs cultured at pH 6.5 were treated with MDL-12, the elevated amplitude of rhythms observed at pH 6.5 was suppressed (Figure 6A). Notably, this occurred in a dose-dependent manner, indicating that this is a drug-dependent effect. Importantly, rhythms of MDL-12-treated macrophages at pH 7.4 had similar amplitude to vehicle-treated macrophages at pH 7.4. This suggests that the inhibitory effect of MDL-12 on pH-induced enhancement of amplitude in macrophage rhythms was specific to signaling occurring under acidic conditions. However, MDL-12 treatment of macrophages at pH 7.4 resulted in shortened period and decreased damping rate compared to vehicle-treated macrophages, suggesting that MDL-12 treatment

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had additional effects on rhythms. Despite this, MDL-12-mediated suppression of pH-driven changes in amplitude, but not period or damping, suggests that the pH-driven changes in these different parameters of rhythms may occur through different pathways. Interestingly, although the adenylyl cyclase inhibition by MDL-12 is reported to be irreversible, we found that pretreatment up to 2 hours is not sufficient to suppress pH-induced changes in amplitude. Only when macrophages continued to be cultured with MDL-12 while exposed to acidic conditions was amplitude suppressed (Supplementary Figure 5). Meanwhile, co-treating cells with acidic pH and MDL12 without any pre-treatment was sufficient to suppress elevation of amplitude under acidic conditions (Figure 6A).

Evidence suggests that acidic pH signals through the cAMP pathway to promote a pro-resolution phenotype in macrophages, with induction of *Icer* occurring directly downstream of cAMP signaling[29]. Despite preventing changes in amplitude under acidic pH, MDL12 treatment at the dose and treatment schedule used does not suppress induction of *Icer* in macrophages under acidic conditions (Figure 6B). However, induction of *Arg1* expression in macrophages under acidic conditions was suppressed by MDL-12. This suggests that at the dose and treatment strategy used, MDL-12 partially suppresses the response of macrophages to acidic pH by suppressing the pH-driven polarization toward a pro-resolution phenotype and changes in amplitude.

To further investigate how MDL-12 was influencing cAMP signaling at the dose and treatment strategy used, we evaluated phosphorylation of cyclic AMP-response element binding protein (CREB). Phosphorylation of CREB occurs downstream of cAMP and has commonly been used as a readout to assess induction of cAMP production in macrophages[78, 82]. In line with evidence in the literature that exposure to acidic pH drives an increase in intracellular cAMP in macrophages[29], we observed that downstream phosphorylation of CREB was elevated in macrophages exposed to acidic pH compared to

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348 those in non-acidic conditions (Figure 6C). Unexpectedly, pCREB levels remained elevated in BMDMs at
349 pH 6.5 despite treatment with MDL-12, indicating that pH-driven phosphorylation of CREB was not
350 suppressed by MDL12 treatment. In fact, pCREB was elevated in MDL-12-treated BMDMS at pH 7.4,
351 suggesting that MDL-12 treatment alone induced phosphorylation of CREB. This is particularly surprising
352 considering that amplitude was not altered in MDL-12-treated macrophages at neutral pH 7.4 despite
353 elevated pCREB. This suggests that some elements of the cAMP signaling pathway, such as pCREB, may
354 be divorced from the pH-induced changes in rhythms. Collectively, our data indicate that while the cAMP
355 signaling pathway is induced under acidic conditions, pH-induced changes in rhythms may not be
356 attributed to cAMP signaling alone, as MDL-12 treatment suppressed pH-induced changes in amplitude
357 of rhythms, but not period or damping, without suppressing signaling through the cAMP pathway.
358
359 **There is evidence of circadian disorder in tumor-associated macrophages, which may be due to**
360 **heterogeneity in circadian rhythms within the TAM population.**
361
362 As we have observed that acidic pH at levels commonly observed in the TME can alter circadian rhythms
363 in macrophages *in vitro* and *ex vivo*, we next sought to investigate whether circadian rhythms can be
364 altered in the TME *in vivo*. Using publicly available data, we analyzed gene expression of tumor-
365 associated macrophages isolated from LLC (Lewis Lung carcinoma) tumors[83]. In line with previous
366 observations, TAMs had elevated expression of *Arg1* relative to peritoneal macrophages (Figure 7A).
367 Expression of *Crem*, which encodes *Icer*, was also elevated in TAMs, indicating that these TAMs were
368 exposed to acidic conditions within the TME (Figure 7A)[29].
369
370 To understand the status of the circadian clock in TAMs, we performed clock correlation distance (CCD)
371 analysis. This analysis has previously been used to assess functionality of the circadian clock in whole

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tumor and in normal tissue[84]. As the circadian clock is comprised of a series of transcription/translation feedback loops, gene expression is highly organized in a functional, intact clock, with core clock genes existing in levels relative to each other irrespective of the time of day. In a synchronized population of cells, this ordered relationship is maintained at the population level, which can be visualized in a heatmap. Using previously published RNA-sequencing data of mouse peritoneal macrophages[44], we found that gene co-expression of clock genes was ordered in a population of wild type macrophages with functional clocks and intact circadian rhythms (Figure 7B). In contrast, clock gene co-expression is disordered in BMAL1 KO macrophages with a genetic disruption of the circadian clock, leading to disruption of circadian rhythms (Supplementary Figure 6). Clock correlation distance analysis revealed that, similar to the BMAL1 KO peritoneal macrophages, the co-expression relationship between the core circadian clock genes in TAMs is significantly more disordered than that of WT peritoneal macrophages (Figure 7C). This indicates that there is population-level disorder in the circadian rhythms of tumor-associated macrophages, suggesting that circadian rhythms are indeed altered in macrophages within the TME.

Circadian disorder assessed by CCD has previously been used to infer disruption of circadian rhythms[85]. Indeed, we observed that genetic disruption of circadian rhythms by BMAL1 KO resulted in a disordered clock, as observed in peritoneal macrophages (Figure 7B). However, since CCD is a population-level analysis, heterogeneity of rhythms, as observed in a desynchronous cell population, rather than disruption of rhythms, may also underlie the circadian disorder observed by CCD. Heterogeneity in macrophage phenotype, exposure to acidic pH, and lactate are all factors present in the TME and relevant to tumor-associated macrophages. We have observed that each of these factors can alter circadian rhythms in macrophages, both alone and in combination with each other. Thus, we sought

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to understand if heterogeneity in macrophage rhythms could be contributing to the disorder in clock gene co-expression and poor CCD score indicative of population-level disorder in TAM rhythms.

To address this, we examined if differences in rhythms of macrophages within a population might contribute to population-level disorder as measured by CCD. To this end, we used publicly available data of peritoneal macrophages taken at different times of day in four-hour intervals across two days[36]. We then constructed four different sample groups in which samples were pooled according to time of day of harvest. As a control population for synchronized cell population with homogenous rhythms, samples taken at the same time of day were pooled. We then modeled a progressively desynchronized population with increased differences in phase of rhythms by pooling samples that were taken four hours apart, eight hours apart, or twelve hours apart (Figure 8A). CCD was then performed on these four populations (Figure 8B). CCD score worsened as populations became increasingly desynchronized, with the 12hr desynchronized population having a significantly worse CCD score than synchronized, homogenous macrophage population (Figure 8C). This indicates that as circadian rhythms of individual macrophages within a population become more different from each other, circadian disorder increases at the population-level. This analysis suggests that heterogeneity in rhythms, as observed with desynchrony, may underlie population-level disorder of the circadian clock as measured by CCD. Collectively, these data suggest that rhythms are disordered in tumor-associated macrophages, and that heterogeneity in macrophage rhythms within the TAM population may underlie this population-level disorder in circadian rhythms of TAMs observed by CCD.

Circadian rhythms of macrophages can influence tumor growth in a murine model of pancreatic cancer.

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We then sought to determine how circadian rhythms in tumor-associated macrophages may influence tumor growth in KCKO, which is a murine model of pancreatic ductal adenocarcinoma (PDAC)[86, 87]. To this end, we used a genetic disruption of the circadian clock in macrophages. While myeloid-specific genetic mouse models are useful, they are not macrophage-specific; thus, co-injection experiments are commonly used to determine macrophage-specific roles[77, 88, 89]. Thus, we co-injected BMDMs from WT or BMAL1 KO mice along with KCKO cells into WT mice, and tumor growth was measured. We saw a significant increase in the growth of tumors co-injected with BMAL1 KO macrophages compared to those co-injected with WT macrophages (Figure 9). These results suggest intact circadian rhythms of macrophages can restrain tumor growth, in agreement with similar published findings in a murine model of melanoma[88].

Discussion

Macrophages experience altered environmental conditions within the tumor microenvironment, but how these may affect macrophage circadian rhythms remains unclear. Here we assessed whether circadian rhythms are altered in macrophages within the TME. To this end, we explored whether conditions commonly associated with the tumor microenvironment could influence circadian rhythms in macrophages. As TAMs are phenotypically heterogenous, we first assessed circadian rhythms in macrophages polarized toward different phenotypes. We found that polarization state affects circadian rhythmicity, with pro-inflammatory macrophages exhibiting far weaker rhythms that pro-resolution macrophages (Figure 1). We then modeled acidic conditions in the TME by exposing macrophages to pH and lactate levels similar to those found in the TME, and found that low pH in particular dramatically altered the rhythms of macrophages (Figures 2-4). Changes in cAMP signaling may contribute to these changes in rhythmicity, but low pH induced alterations far beyond what is observed by enhancing cAMP

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443 signaling pharmacologically (Figure 5). While the adenylyl-cyclase inhibitor MDL-12 largely rescues the
444 changes in amplitude observed in low pH, our data suggest that a pathway other than canonical cAMP
445 signaling may be involved in this effect (Figure 6). Finally, we assessed the status of the circadian clock in
446 tumor-associated macrophages, the potential contribution of heterogeneity in circadian rhythms to
447 population-level rhythms, and assessed whether the circadian regulation of macrophages impacts tumor
448 growth. Our results indicate that macrophage rhythms as a whole are disordered within tumors (Figure
449 7), and that heterogeneity in rhythms within the tumor-associated macrophage population may underlie
450 this observed circadian disorder (Figure 8). We further demonstrated that the intact macrophage
451 circadian clock can suppress tumor growth (Figure 9). Overall, our results for the first time demonstrate
452 that exposure of macrophages to conditions associated with the tumor microenvironment can influence
453 circadian rhythms, a key aspect of macrophage biology.
454
455 A critical question in understanding the role of circadian rhythms in macrophage biology is determining
456 how different polarization states of macrophages affect their internal circadian rhythms. This is
457 especially important considering that tumor-associated macrophages are a highly heterogeneous
458 population. Our data indicate that compared to unstimulated macrophages, rhythms are enhanced in
459 pro-resolution macrophages, characterized by increased amplitude and improved ability to maintain
460 synchrony; in contrast, rhythms are suppressed in pro-inflammatory macrophages, characterized by
461 decreased amplitude and impaired ability to maintain synchrony (Figure 1). These agree with the
462 findings of others which have shown that polarizing stimuli alone and in combination with each other
463 can alter rhythms differently in macrophages[64, 65]. In a tumor, macrophages exist along a continuum
464 of polarization states and phenotypes[18-21, 24]. Thus, while our characterizations of rhythms in *in vitro*-
465 polarized macrophages provide a foundation for understanding how phenotype affects circadian
466 rhythms of macrophages, further experiments will be needed to assess macrophages across the full

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spectrum of phenotypes. Indeed, alteration of rhythms may be just as highly variable and context-dependent as phenotype itself.

In addition to polarizing stimuli, tumor-associated macrophages are exposed to a variety of conditions within the tumor microenvironment that may alter their circadian rhythms. We observed that exposure to acidic pH alters rhythms in macrophages, increasing amplitude of pro-resolution macrophages but suppressing amplitude of pro-inflammatory macrophages (Figure 2). This indicates that pH affects rhythms differently depending on phenotype, hinting at additional layers of complexity in how the environment could contribute to changes in circadian rhythms. Even further changes in rhythms were observed when macrophages were exposed to lactate in conjunction with acidic pH (Figure 4). These observations suggest that the combination of stimuli present in the microenvironment such as lactate and low pH, as well as various polarizing stimuli, can each contribute to modulate rhythms, resulting in highly context-dependent changes in circadian rhythms of macrophages based on the microenvironment. As macrophages are highly plastic and are exquisitely capable of sensing and responding to their environment, one could reason that changes in circadian rhythms, and downstream circadian regulation, are a mechanism by which macrophages can adopt different programs to respond to their environment.

It was previously observed that acidic pH can disrupt circadian rhythms in cell lines[32]. However, while acidic pH alters rhythms in macrophages, it does not ablate them. This suggests that the influence of acidic pH on circadian rhythms can vary between cell types. pH-induced circadian disruption was found to be driven by inhibition of mTORC1 activity in cell lines, and there was evidence to suggest that mTORC1 activity was sensitive to pH in T cells [90]. Thus, the role of mTORC1 activity in mediating pH-driven changes in circadian rhythms of macrophages will be a topic of future investigation.

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The mechanism through which acidic pH can modulate the circadian clock in macrophages remains unclear. Evidence in the literature suggests that acidic pH promotes a pro-resolution phenotype in macrophages by driving signaling through the cAMP pathway[29]. It has previously been shown that cAMP signaling can modulate the circadian clock[81]. However, our data indicate that cAMP signaling is not fully sufficient to confer pH-mediated changes in circadian rhythms in macrophages. Treatment with forskolin or IBMX enhanced amplitude of oscillation, but was not sufficient to fully recapitulate pH-induced changes in rhythms (Figure 5). Interestingly, treatment with MDL-12, commonly known as an inhibitor of adenylyl cyclase[29, 91], resulted in suppression of pH-induced changes in amplitude of circadian rhythms and alleviated pH-mediated polarization toward a pro-resolution phenotype, measured by *Arg1* expression (Figure 6). However, our data indicate that treatment with MDL-12 at the dose and treatment strategy used did not inhibit signaling through the cAMP signaling pathway, as *Icer* induction and phosphorylation of CREB were not suppressed. While MDL-12 is commonly used as an adenylyl cyclase inhibitor, it has also been documented to have inhibitory activity toward phosphodiesterases (PDEs) and the import of calcium into the cytosol through various mechanisms[92, 93]. This is of particular interest, as calcium signaling has also been shown to be capable of modulating the circadian clock[94]. Furthermore, while acid-sensing through GPCRs have been the most well-characterized pathways in macrophages, there remain additional ways in which acidic pH can be sensed by macrophages such as acid-sensing ion channels[95, 96]. Further work is required to understand the signaling pathways through which pH can influence macrophage phenotype and circadian rhythms.

Taken together, our findings indicate that both macrophage polarization state and exposure to environmental conditions such as low pH and lactate each affect the circadian rhythms of macrophages. One prominent question that remains elusive is whether circadian rhythms of macrophages are

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515 governed by phenotype, or whether phenotype is subject to control of circadian rhythms. Alternately,
516 the relationship between the two could very well be bi-directional. Acidic pH promotes a pro-resolution
517 phenotype and suppresses pro-inflammatory activity as early as 2 hours post-exposure (Supplementary
518 Figure 3), with changes in rhythms observed by 12 hours (Figure 2). This suggests that pH-driven changes
519 in phenotype may precede changes in rhythms. How much the pH-driven changes in rhythms is
520 influenced directly by pH sensing, or indirectly through the influence of pH on phenotype, remains
521 unknown. Certainly, polarization toward a pro-resolution phenotype and exposure to acidic pH each
522 have different effects on circadian rhythms of macrophages; while polarization of macrophages toward a
523 pro-resolution phenotype increases amplitude and decreases damping rate of circadian rhythms,
524 exposure to acidic pH not only increases amplitude, but shortens period and increases damping rate of
525 rhythms (Figures 1, 2). Additionally, acidic pH drives changes in rhythms of pro-resolution macrophages
526 similar to that of unstimulated macrophages, suggesting that the signaling driving these changes in
527 rhythms are not redundant between the two conditions. This suggests that acidic pH-promoted
528 polarization toward a pro-resolution phenotype does not on its own account for the pH-driven changes
529 in rhythms. Studies have shown that circadian rhythms are regulated by metabolism[97], and it is well-
530 appreciated that macrophages of distinct phenotypes prioritize different metabolic pathways[98]. Thus,
531 the metabolic needs of macrophages of different phenotypes likely influences the response to pH and
532 other metabolically stressful conditions in the TME, and downstream effects on circadian rhythms.
533 Indeed, we observed that exposure to acidic pH had an opposite effect on rhythms of pro-inflammatory
534 macrophages compared to pro-resolution macrophages (Figure 2). Further work is required to
535 understand the interplay between macrophage phenotype, metabolism, and circadian rhythms.
536
537 We observed that acidic pH appears to enhance circadian rhythms of unstimulated and pro-resolution
538 macrophages, and we and others have shown evidence that macrophages are exposed to an acidic

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environment within the TME[28, 29]. However, analysis of TAMs by clock correlation distance (CCD) presents evidence that rhythms are disordered in TAMs (Figure 7). CCD is one of the most practical tools currently available to assess circadian rhythms due to its ability to assess rhythms independent of time of day and without the need for a circadian time series, which is often not available in publicly available data from mice and humans[84]. However, CCD has some limitations, primary of which is that it is a measure of population-level circadian rhythms. There is currently a major barrier in the circadian field in the ability to measure circadian rhythms at the single-cell level. Some recent work has defined single-cell rhythms and uncovered a surprising amount of heterogeneity in mouse and fly tissues[99-101]**.** A full measure of single-cell circadian dynamics within a tissue must be assessed to facilitate studies of rhythms *in vivo* where cell populations are highly heterogeneous, such as within the tumor microenvironment or at a site of infection. Indeed, we present evidence that heterogeneity of circadian rhythms within a given population may underlie this observed circadian disorder (Figure 8). We have shown that various stimuli can alter rhythms of macrophages in a complex and contributing manner, including polarizing stimuli, acidic pH, and lactate. TGFβ is produced by a variety of cells within the TME, and was recently identified as a signal that can modulate circadian rhythms[102, 103]. Thus, it is conceivable that, in addition to acidic pH, other stimuli in the TME are influencing circadian rhythms to drive population-level disorder that we are observing by CCD. Notably, we cannot rule out that the disorder observed by CCD may be indicative of circadian disruption.

In light of our observations that conditions associated with the TME can alter circadian rhythms in macrophages, it becomes increasingly important to understand the relevance of macrophage rhythms to their function in tumors. Data from our lab and others suggest that disruption of the macrophage-intrinsic circadian clock accelerates tumor growth, indicating that circadian regulation of macrophages is tumor-suppressive[88]. This agrees with complementary findings that behavioral disruption of circadian

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563 rhythms in mice (through chronic jetlag) disrupts tumor macrophage circadian rhythms and accelerates
564 tumor growth[56]**.** It remains unclear whether this is through the pro-tumorigenic functions of
565 macrophages such as extracellular matrix remodeling or angiogenesis, through suppression of the anti-
566 tumor immune response, or a combination of both functions. Further work will be needed to tease apart
567 these distinctions.
568
569 Whereas much work has been done to characterize how macrophages are regulated within the TME, the
570 impact of the TME on circadian rhythms of macrophages remained elusive. Our work uncovers a novel
571 way in which conditions associated with the TME can influence macrophage biology through modulation
572 of circadian rhythms. While the majority of studies investigating the circadian regulation of macrophages
573 have been conducted studying macrophages under homeostatic conditions or in response to acute
574 inflammation[36, 38, 44, 45, 104], our work contributes to an emerging body of evidence that the tissue
575 microenvironment can influence circadian rhythms[102]. This is increasingly important when considering
576 the role of circadian rhythms in immune responses at sites of ongoing, chronic inflammation where the
577 microenvironment is altered, such as within tumors. In identifying factors within the TME that can
578 modulate circadian rhythms of macrophages and uncovering evidence of circadian disorder within
579 tumor-associated macrophages, this work lays the foundation for further studies aimed at understanding
580 how the TME can influence the function of tumor-associated macrophages through modulation of
581 circadian rhythms.
582
583 **Limitations of the Study**
584
585 Our observations of rhythms in macrophages of different phenotypes are limited by *in vitro* polarization
586 models. It is important to note that while our data suggest that pro-inflammatory macrophages have

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suppressed rhythms and increased rate of desynchrony, it remains unclear the extent to which these findings apply to the range of pro-inflammatory macrophages found *in vivo*. We use IFNγ and LPS co-treatment *in vitro* to model a pro-inflammatory macrophage phenotype that is commonly referred to as 'M1', but under inflammatory conditions *in vivo*, macrophages are exposed to a variety of stimuli that result in a spectrum of phenotypes, each highly context-dependent. The same is true for for 'M2'; different tissue microenvironment are different and pro-resolution macrophages exist in a spectrum. Rhythms were heavily suppressed in pro-inflammatory macrophages, which makes analysis of rhythm parameters in pro-inflammatory macrophages more challenging as amplitude and signal reaches limit of detection. We assessed parameters of oscillation using LumiCycle Analysis, a method commonly used in the circadian field[64, 105]. Our observations of changes in amplitude and period in pro-inflammatory macrophages compared to unstimulated macrophages agrees with the literature, where these changes in rhythms have been observed using LumiCycle as well as by mRNA[64, 65]. This supports the validity and reproducibility of our observations despite the challenges of observing and analyzing rhythms of pro-inflammatory macrophages.

Methods

Animals

Mice were maintained in individually ventilated cages with bedding and nesting material in a temperature-controlled, pathogen-free environment in the animal care facility at the University of Rochester. All animal protocols were approved by the University of Rochester Committee of Animal Resources (UCAR). All experiments were performed in compliance with the NIH- and University of Rochester-approved guidelines for the use and care of animals, as well as recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council[106]. Mice were housed on a 12:12 light dark cycle. In some cases, to ease timepoint collection, mice were housed under reverse

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611 lighting conditions in a 12:12 dark light cycle for at least 2 weeks prior to use in experiments. Mice used
612 for experiments were between the ages of 8-14 weeks old; both male and female mice were used. Mice
613 were euthanized humanely prior to harvesting peritoneal macrophages or bone marrow.
614
615 Previously characterized mice with a myeloid-specific deletion of BMAL1 (LysM-cre$^{+/-}$Bmal1$^{flox/flox}$;
616 referred to as BMAL1 KO mice)[45] in a C57BL/6 background were generated by crossing LysM-cre
617 mice[107] with Bmal1$^{flox/flox}$ mice[108]. These mice were further crossed with PER2-Luc mice[61] to
618 generate BMAL1 KO or wild-type control mice (LysM-cre$^{-/-}$Bmal1$^{flox/flox}$; referred to as WT) expressing
619 PER2-Luc. PER2-Luc (strain #006852), LysM-cre (strain #004781), and Bmal1$^{flox/flox}$(strain #007668) mice
620 used for breeding to generate WT and BMAL1 KO mice were purchased from the Jackson Laboratory.
621
622 **Differentiation and culture of bone marrow-derived macrophages**
623 Bone marrow-derived macrophages (BMDMs) were generated from bone marrow isolated from WT
624 mice using a well-established protocol for differentiation of BMDMs over 7 days[109, 110]. In brief, bone
625 marrow cells were seeded at 200,000 cells/mL on non-tissue culture treated-plates in BMDM
626 Differentiation Media: RPMI (Corning, CAT#MT10040CV) supplemented with 20% (v/v) L929 supernatant
627 and 10% (v/v) heat-inactivated (HI) fetal bovine serum (FBS) (Cytiva, CAT#SH30396.03), supplemented
628 with 100 U/mL Penicillin-Streptomycin (Gibco, CAT# 15140122). Cells were grown at 37^{o}C in air enriched
629 with 5% CO_2. On day 3, additional BMDM Differentiation Media was added to cells. On day 6 of the
630 differentiation protocol, BMDMs were seeded at 1.2*10^{6} cells/mL and left in BMDM Differentiation
631 Media, and kept at 37^{o}C in air enriched with 5% CO_2. On day 7, BMDM Differentiation Media was
632 removed and BMDMs were synchronized.
633

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To synchronize BMDMs, BMDMs were first serum starved for 24 hours in serum-free media (RPMI, supplemented with 100 U/mL Penicillin-Streptomycin); BMDMs were then subjected to serum shock by replacing serum-free media with RPMI supplemented with 50% (v/V) HI horse serum (Corning, CAT#35030CV) at 37^{o}C in air enriched with 5% CO_2. At the end of this synchronization protocol, media was replaced with Atmospheric Media, which has been formulated for use at atmospheric CO_2 levels and enhanced pH stability by increasing buffering capacity at low pH[32]: RPMI (Corning, CAT#50-020-PC), 25mM HEPES (Gibco, CAT#15630080), 25mM PIPES (Sigma, CAT#P1851), supplemented with 10% (v/v) HI FBS and 100 U/mL Penicillin-Streptomycin. Atmospheric Media was adjusted to pH 7.4, 6.8, or 6.5 with NaOH and filter-sterilized.

BMDMs cultured in Atmospheric Media at pH 7.4, 6.8, or 6.5 were either left unstimulated or were polarized toward a pro-resolution ('M2') or pro-inflammatory phenotype ('M1') by addition of 10 ng/mL IL-4 (PeproTech, CAT#214-14) and 10 ng/mL IL-13 (PeproTech, CAT# 210-13), or 50 ng/mL IFNγ (PeproTech, CAT#315-05) and 100 ng/mL LPS (Invitrogen, CAT#00497693), respectively. For lactate experiments, sodium-L-lactate (Sigma, CAT#L7022) or vehicle was added to Atmospheric Media for 25 mM sodium-L-lactate or 0 mM sodium-L-lactate in Atmospheric Media. For interrogation of cAMP signaling pathway, BMDMs were cultured in Atmospheric Media at pH 7.4 or 6.5 with vehicle or 5, 10, or 15 µM MDL-12330A (Sigma, CAT#M182). For phenocopy experiments (Figure 5), BMDMs were not synchronized prior to the experiment. BMDMs were cultured in Atmospheric Media at pH 7.4 or 6.5 with vehicle or 20, 40, or 80 µM IBMX (Sigma, CAT#I5879) or forskolin (Sigma, CAT#344270). For LumiCycle experiments, 100mM D-luciferin was added to Atmospheric Media at 1:1000 for 100µM D-luciferin (Promega, CAT#E1602). Cells cultured in Atmospheric Media were kept at 37^{o}C in atmospheric conditions, and were either monitored over time by LumiCycle or harvested for RNA or protein at the time points indicated.

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658

659 **Isolation and culture of peritoneal macrophages**

660 Peritoneal exudate cells were harvested from mice as previously published[111]. To isolate peritoneal
661 macrophages, peritoneal exudate cells were seeded at $1.2*10^6$ cells/mL in RPMI/10% HI FBS
662 supplemented with 100U/mL Penicillin-Streptomycin and left at 37°C for 1 hour, after which non-
663 adherent cells were rinsed off[110]. Peritoneal macrophages were then cultured in Atmospheric Media
664 at pH 7.4 or 6.5 with 100µM D-luciferin, and kept at 37°C in atmospheric conditions.

665

666 **Quantification of circadian rhythm parameters**

667 Using the Lumicycle Analysis program version 2.701 (Actimetrics), raw data was fitted to a linear
668 baseline, and the baseline-subtracted data was fitted to a damped sin wave from which period and
669 damping were calculated[105]. Amplitude was calculated from baseline-subtracted data by subtracting
670 the bioluminescent values of the first peak from the first trough as previously published[64].

671

672 **Quantitative PCR**

673 Cells were lysed and RNA was isolated using the E.Z.N.A. HP Total RNA Kit (Omega BioTek, CAT#R6812-
674 02). RNA was reverse transcribed to cDNA using the ABI Reverse Transcription Reagents system, using
675 oligo dT for priming (Applied Biosystems, CAT#N8080234). qPCR was performed with cDNA using
676 PerfeCTa SYBR Green FastMix (QuantaBio, CAT#95074-05K) and with the Quant Studio 5 quantitative PCR
677 machines (Applied Biosystems). Triplicate technical replicates were performed, outlier replicates (defined
678 as being more than 1 Ct away from other two replicates) were discarded, and relative mRNA was
679 normalized to *Tbp* and assessed by the ΔΔCt. The following primers were used: *Tbp* (5'-
680 CCAGAACTGAAAATCAACGCAG-3', 5'-TGTATCTACCGTGAATCTTGGC-3') (IDT, Mm.PT.39a.22214839), *Arg1*
681 (5'-CTCCAAGCCAAAGTCCTTAGAG-3', 5'-AGGAGCTGTCATTAGGGACATC-3')[112], *Vegf* (5'-

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CCACGACAGAAGGAGAGCAGAAGTCC-3', 5'-CGTTACAGCAGCCTGCACAGCG-3')[77], *Nos2* (5'-GCTTCTGGTCGATGTCATGAG-3', 5'-TCCACCAGGAGATGTTGAAC-3'), *Icer* (5'-ATGGCTGTAACTGGAGATGAA-3', 5'-GTGGCAAAGCAGTAGTAGGA-3')[29], *Per2* (5'-TGAGGTAGATAGCCCAGGAG-3,' 5'-GCTATGAAGCGCCTAGAATCC-3') (IDT, Mm.PT.58.5594166), *Cry1* (5'-GCTATGCTCCTGGAGAGAACG T-3,' 5'-TGTCCCCGTGAGCATAGTGTAA-3')[113], *Nr1d1* (5'-GAGCCACTAGAGCCAATGTAG-3,' 5'-CCAGTTTGAATGACCGCTTTC-3') (IDT, Mm.PT.58.17472803).

Western blot

Cells were lysed using the M-Per lysis reagent (Thermo Scientific, CAT#78501), supplemented with protease and phosphatase inhibitor cocktail (1:100; Sigma, CAT#PPC1010) and phosphatase inhibitor cocktail 2 (1:50; Sigma, CAT#P5726), with 200µM deferoxamine (Sigma, CAT#D9533). Lysates were incubated on ice for 1 hour, then centrifuged at 17,000 xg to pellet out debris; supernatant was collected. Protein was quantified using the Bio-Rad DC Protein Assay Kit (Bio-Rad, CAT#5000112), and lysates of equal concentration were prepared and run by SDS-PAGE on Bio-Rad Criterion 4–15% 26-well gradient gel (Bio-Rad, CAT#5678095). Gels were transferred using the Trans-Blot Turbo system (Bio-Rad) to nitrocellulose membranes (Bio-Rad CAT#1704271).

The following primary antibody was used: rabbit anti-p-CREB (Ser133, Ser129) (Invitrogen, CAT#44-297G). The following secondary antibody was used: goat anti-rabbit Alexa Fluor 680 (Invitrogen, CAT#A21109). Of note, two different anti-CREB antibodies were tested (Cell Signaling, CAT#9197 and Invitrogen, CAT#35-0900) in combination with the appropriate secondary antibody, but neither revealed bands at the correct molecular weight for CREB protein. Membranes were digitally imaged using a ChemiDoc MP (Bio-Rad) and uniformly contrasted. Total protein was imaged by Stain-Free imaging

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technology (Bio-Rad) and used as loading control. To visualize total protein, image of entire membrane was shrunk to match the size of pCREB.

Survival under acidic pH

BMDMs were seeded, in triplicate, at $1.2*10^6$ cells/mL in a 96-well plate. BMDMs were synchronized, then cultured in Atmospheric Media at pH 7.4, 6.8, or 6.5 containing 10 ng/mL IL-4 and 10 ng/mL IL-13, or 50 ng/mL IFNγ and 100 ng/mL LPS, or vehicle for unstimulated control. BMDMs were fixed at 1, 2, and 3 days later. BMDMs were stained with DAPI, and plates were imaged using a Celigo S. Number of nuclei per well was enumerated using Celigo software to quantify the number of adherent BMDMs after time in culture under acidic conditions as a readout of survival.

Tumor growth

Mice were anesthetized via inhalation of 4 vol% isoflurane in 100% oxygen at a flow rate of 4 L/min prior to injection. Following application of 70% ethanol to the site of injection, with $1*10^6$ WT or BMAL1 KO macrophages and $1*10^6$ KCKO cells in 100 uL saline were subcutaneously co-injected in the flank of WT mice. In line with previously published co-injection tumor experiments, mice were injected with macrophages at a 1:1 ratio[77, 88]. Tumor growth was measured by caliper, and volume was calculated by the modified ellipsoidal formula: tumor volume = $0.5*(\text{length}*\text{width}^2)$[114]. Mice were euthanized when there was ulcer formation or when tumor size reached a diameter of 20mm.

Processing and analysis of publicly available gene expression data

FASTQ files from a previously published analysis of peritoneal macrophages from WT or BMAL1 KO mice were downloaded from EMBL- European Bioinformatics Institute Array Express (accession #E-MTAB-8411)[44]. For these samples, multiple files of the same run were concatenated before mapping. FASTQ

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files from a previous published study of tumor-associated macrophages were downloaded from NCBI GEO (accession #GSE188549)[83]. Both sets of samples were mapped to transcripts using Salmon 1.3.0 in mapping-based mode using a decoy-aware transcription built from the Gencode M25 GRCm38 primary assembly mouse genome and M25 mouse transcriptome[115]. Single-end mapping was used for GSE188549 samples and paired-end mapping was used for E-MTAB-8411. All transcripts were then collapsed to gene-level using Tximport v1.14.2with the Gencode M25 transcriptome, and genes were annotated with symbols using the Ensembl GRCm38.100 transcriptome annotations[116]. Transcripts per million (TPM) outputted from Tximport were used for downstream analyses. CEL files from a previously published time series analysis of peritoneal macrophages from WT mice were downloaded from NCBI GEO (accession #GSE25585)[36]. Microarray data was imported and analyzed from CEL files using the packages affy and Limma, and genes were annotated with symbols using the University of Michigan Brain Array Custom CDF v25.0 for the Mouse Gene 1.0 ST Array[117, 118]. Clock correlation distance analysis was performed as previously described[84].

Data sharing

Raw data will be provided by uploading to FigShare, as will R code used for data analysis.

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Conflict of Interest

The authors declare no conflict of interest.

Figure Legends

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775 **Figure 1. Macrophages of different phenotypes have distinct circadian rhythms.** Bone marrow-derived
776 macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. The circadian clocks of
777 BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed
778 by a 2-hour period of serum shock in media with 50% serum. Luciferase activity of BMDMs stimulated
779 with either 10 ng/mL IL-4 and 10 ng/mL IL-13, or 50 ng/mL IFNγ and 100 ng/mL LPS; or left unstimulated
780 was monitored in real time by LumiCycle. Data was baseline-subtracted using the running average.
781 Oscillation parameters of BMDMs were measured by LumiCycle Analysis. Shown are mean and standard
782 error of the mean (SEM), n=5 biological replicates. Statistical significance determined by unpaired two-
783 tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$. Data shown are
784 representative of 2 independent experiments.
785
786 **Figure 2. Acidic pH alters circadian rhythms of bone marrow-derived macrophages *in vitro*.** Bone
787 marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. The
788 circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0%
789 serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then
790 cultured in media with neutral pH 7.4 or acidic media with pH 6.8 or 6.5, and stimulated with either 10
791 ng/mL IL-4 and 10 ng/mL IL-13 (B, E), or 50 ng/mL IFNγ and 100 ng/mL LPS (C, F); or left unstimulated (A,
792 D). Luciferase activity was monitored in real time by LumiCycle (A, B, C). Shown are mean and SEM, n=2
793 biological replicates, representative of 2 independent experiments. Data from both experiments was
794 baseline-subtracted using the running average, and oscillation parameters were measured by LumiCycle
795 Analysis. Shown are mean and SEM, n=5 biological replicates. In parallel, RNA was collected at 12, 16, 20,
796 and 24 hours post-synchronization, and qt-PCR was performed to assess oscillation of transcripts
797 encoding core clock proteins in macrophages under acidic conditions (D, E, F). Shown are mean and SEM,
798 n=3 biological replicates. Data shown are representative of 2 independent experiments. Statistical

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799 significance determined by unpaired two-tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$;
800 ***, $p<0.0005$.
801
802 **Figure 3. Acidic pH alters circadian rhythms of bone marrow-derived macrophages *ex vivo* at**
803 **temporally distinct times of day.** Peritoneal macrophages were obtained at ZT0 or ZT12 from C57BL/6
804 mice expressing PER2-Luc and cultured in media with neutral pH 7.4 or acidic pH 6.5. Luciferase activity
805 was monitored in real time by LumiCycle (A). Shown are mean and SEM, n=2 biological replicates,
806 representative of 2 independent experiments. Data from both experiments was baseline-subtracted
807 using the running average, and oscillation parameters were measured by LumiCycle Analysis; shown are
808 mean and SEM, n=4 biological replicates. Statistical significance determined by unpaired two-tailed T test
809 with Welch's correction. The magnitude of change in circadian oscillation parameters between
810 macrophages at pH 7.4 and pH 6.5 was compared between peritoneal macrophages taken at ZT 0 or ZT
811 12 (B). Shown is the mean and SEM, n=4; data pooled from the 2 independent experiments in (A).
812 Statistical significance determined by paired two-tailed T test with Welch's correction; *, $p < 0.05$; **,
813 $p<0.005$; ***, $p<0.0005$.
814
815 **Figure 4. Lactate alters circadian rhythms in macrophages, both alone and in conjunction with acidic**
816 **pH.** Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-
817 Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media
818 with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were
819 then cultured in media with neutral pH 7.4 or acidic pH 6.5, supplemented with 0 mM or 25 mM sodium-
820 L-lactate. RNA was collected at 6 hours post-treatment, and expression of pro-resolution phenotype
821 markers *Arg1* or *Vegf* was quantified by qtPCR (A). Shown is the mean and SEM, n=6 biological replicates,
822 data pooled from 2 independent experiments. Luciferase activity was monitored in real time by

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LumiCycle (B). Shown is the mean and SEM, n=4 biological replicates; data representative of 2 independent experiments. Data from both experiments was baseline-subtracted using the running average, and oscillation parameters were measured by LumiCycle Analysis. Shown is the mean and SEM, n=7-10 biological replicates. Statistical significance determined by unpaired two-tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$; ****, $p<0.00005$.

Figure 5. Treatment with Forskolin or IBMX does not fully recapitulate pH-driven changes in circadian rhythms of macrophages. Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. BMDMs were cultured in media with neutral pH 7.4 or acidic pH 6.5, and treated with vehicle or 20, 40 or 80 uM Forsokolin (A), or treated with vehicle or 20, 40, 80 uM IBMX (B). Luciferase activity was monitored in real time by LumiCycle. Data was baseline-subtracted using the running average. Shown is the mean, n=2 biological replicates. Data shown are representative of 2 independent experiments.

Figure 6. Treatment with MDL-12 suppresses pH-induced changes in amplitude of circadian rhythms without suppressing signaling through the cAMP signaling pathway under acidic conditions. Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then cultured in media with neutral pH 7.4 or acidic pH 6.5, and treated with vehicle or 5, 10, or 15uM MDL-12. Luciferase activity was monitored in real time by LumiCycle. Data was baseline-subtracted using the running average, and oscillation parameters were measured by LumiCycle Analysis (A). Shown is the mean, n=4 biological replicates. Expression of genes associated with acid sensing (*Icer*) and pro-resolution phenotype (*Arg1*) was measured (B). Shown is the mean and SEM; n=3 biological replicates.

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Phosphorylation of CREB was assessed (C). Statistical significance determined by unpaired two-tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$; ****, $p<0.00005$. Data shown are representative of 2-3 independent experiments.

Figure 7. Clock correlation distance (CCD) analysis provides evidence of circadian disorder in tumor-associated macrophages. RNAseq datasets of WT peritoneal macrophages (n=5), BMAL1 KO peritoneal macrophages (n=4), and tumor-associated macrophages (TAMs, n=10) were analyzed for expression of *Arg1* and *Crem* (A). Statistical significance determined by one-way ANOVA with Turkey's multiple comparison test; ****, $p<0.00005$. Clock correlation distance (CCD) analysis was performed (B) and statistical analysis to compare CCD scores was performed by calculating delta CCD; *, $p<0.05$ (C).

Figure 8. Heterogeneity in circadian rhythms of cells within a population can lead to circadian disorder observed by CCD. Increasingly desynchronized populations were modeled using an RNAseq data set of WT peritoneal macrophages (n=12) taken at 4-hour intervals across two days. A schematic of the populations used in experimental design (A). Clock correlation distance (CCD) analysis was performed (B) and statistical analysis to compare CCD scores was performed by calculating delta CCD (C).

Figure 9. A functional circadian clock in macrophages can influence tumor growth in a murine model of PDAC. Bone marrow-derived macrophages (BMDMs) obtained from WT or BMAL1 KO mice were subcutaneously co-injected with KCKO cells into the flank of WT mice. Tumor growth was measured by caliper. Shown is the mean and SEM; n=20 individual mice, 10 male and 10 female. Statistical significance determined at each time point by unpaired two-tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$. Data shown are representative of 2 independent experiments.

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Supplemental Figure 1. Polarization of macrophages toward pro-resolution or pro-inflammatory phenotype. Bone marrow-derived macrophages (BMDMs) were derived from C57BL/6 mice expressing PER2-Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then stimulated with either 10 ng/mL IL-4 and 10 ng/mL IL-13, or 50 ng/mL IFNγ and 100 ng/mL LPS; or left unstimulated. RNA was collected at 6 hours post-synchronization, and qt-PCR was performed to assess expression of genes encoding Arginase 1 (*Arg1*) and iNOS (*Nos2*). Shown are mean and standard error of the mean (SEM), n=3 biological replicates. Statistical significance determined by unpaired two-tailed T test with Welch's correction; *, $p < 0.05$; **, ***, $p<0.0005$. Data shown are representative of 2 independent experiments.

Supplemental Figure 2. The PER2-Luciferase reporter system enables real-time monitoring of circadian rhythms of macrophages. A schematic of the PER2-Luciferase (PER2-Luc) luciferase reporter system (A). Bone marrow-derived macrophages (BMDMs) were derived from C57BL/6 mice expressing PER2-Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then cultured in RPMI/10% FBS supplemented with D-luciferin, and luciferase activity was monitored in real time by LumiCycle (B). Shown are mean and SEM; n=2 biological replicates. Data shown are representative of 2 independent experiments.

Supplemental Figure 3. Macrophages sense and respond to an acidic extracellular environment when cultured *in vitro* in media with acidic pH. Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. BMDMs were cultured in media with pH 7.4 or acidic media with pH 6.5, and stimulated with either 10 ng/mL IL-4 and 10 ng/mL IL-13, or 50 ng/mL IFNγ and 100

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ng/mL LPS; or left unstimulated. RNA was collected at 2 hours post-treatment, and qt-PCR was performed to assess expression of genes associated with phenotype (A) or acid sensing (B) in macrophages. Shown are mean and SEM, n=3 biological replicates. Statistical significance determined by two-tailed T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$; ****, $p<0.00005$. Data shown are representative of 2 independent experiments.

Supplementary Figure 4. Survival of macrophages under acidic pH. Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then cultured in media with pH 7.4 or acidic media with pH 6.8 or 6.5, and stimulated with either 10 ng/mL IL-4 and 10 ng/mL IL-13, or 50 ng/mL IFNγ and 100 ng/mL LPS; or left unstimulated. Cells were fixed at 1, 2, and 3 days post-treatment and stained with DAPI. Number of nuclei was counted using Celigo to determine the number of adherent cells. Shown are mean and SEM, n=3 biological replicates. Statistical significance determined by multiple unpaired T test with Welch's correction; *, $p < 0.05$; **, $p<0.005$; ***, $p<0.0005$. Data shown are representative of 2 independent experiments.

Supplementary Figure 5. Pre-treatment with 15 uM MDL-12 for up to 2 hours is not necessary to suppress pH-driven changes in amplitude of circadian rhythms. Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice expressing PER2-Luc. The circadian clocks of BMDMs were synchronized by a 24-hour period of serum starvation in media with 0% serum, followed by a 2-hour period of serum shock in media with 50% serum. BMDMs were then cultured in media with neutral pH 7.4 or acidic pH 6.5, and treated with vehicle or 15uM MDL-12 (A). Alternately, BMDMs were cultured at pH 7.4 and pre-treated with 15uM MDL-12 for 30 minutes (B, C) or 2 hours (D, E), after which media was

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919 removed and cells were cultured in media at pH 7.4 or pH 6.5 in the presence (C, E) or absence (B, D) of
920 MDL-12. Luciferase activity was monitored in real time by LumiCycle. Shown is the mean, n=2 biological
921 replicates. Data for cells that received no pre-treatment (A) was overlaid on plots of data where cells
922 received pre-treatment (B-E) to allow for comparison of changes in rhythms.
923
924 **Supplementary Figure 6. Macrophages from BMAL1 KO mice have disrupted circadian rhythms.** Levels
925 of BMAL1 in bone marrow-derived macrophages (BMDMs) from WT or BMAL1 KO mice were assessed
926 by western blot (A). To confirm functional disruption of the circadian clock, peritoneal macrophages or
927 BMDMs were obtained from WT or BMAL1 KO mice expressing PER2-Luc and cultured in vitro with D-
928 luciferin. Luciferase activity was monitored in real time by LumiCycle (B). Shown is the mean and SEM,
929 n=2, representative of 2 independent experiments.

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References

1. Cassetta, L. and J.W. Pollard, *Targeting macrophages: therapeutic approaches in cancer.* Nat Rev Drug Discov, 2018. **17**(12): p. 887-904.
2. Zhang, Q.W., et al., *Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature.* PLoS One, 2012. **7**(12): p. e50946.
3. Gentles, A.J., et al., *The prognostic landscape of genes and infiltrating immune cells across human cancers.* Nat Med, 2015. **21**(8): p. 938-945.
4. DeNardo, D.G. and B. Ruffell, *Macrophages as regulators of tumour immunity and immunotherapy.* Nat Rev Immunol, 2019.
5. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
6. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
7. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
8. Murray, P.J., *Nonresolving macrophage-mediated inflammation in malignancy.* Febs j, 2018. **285**(4): p. 641-653.
9. Schoppmann, S.F., et al., *Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis.* Am J Pathol, 2002. **161**(3): p. 947-56.
10. Huber, R., et al., *Tumour hypoxia promotes melanoma growth and metastasis via High Mobility Group Box-1 and M2-like macrophages.* Sci Rep, 2016. **6**: p. 29914.
11. Graham, D.K., et al., *The TAM family: phosphatidylserine sensing receptor tyrosine kinases gone awry in cancer.* Nat Rev Cancer, 2014. **14**(12): p. 769-85.
12. Cook, R.S., et al., *MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis.* J Clin Invest, 2013. **123**(8): p. 3231-42.
13. Roberts, A.W., et al., *Tissue-Resident Macrophages Are Locally Programmed for Silent Clearance of Apoptotic Cells.* Immunity, 2017. **47**(5): p. 913-927.e6.
14. Fadok, V.A., et al., *Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.* J Clin Invest, 1998. **101**(4): p. 890-8.
15. Strassmann, G., et al., *Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2.* J Exp Med, 1994. **180**(6): p. 2365-70.
16. Kuang, D.M., et al., *Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1.* J Exp Med, 2009. **206**(6): p. 1327-37.
17. Ruffell, B. and L.M. Coussens, *Macrophages and therapeutic resistance in cancer.* Cancer Cell, 2015. **27**(4): p. 462-72.
18. Mantovani, A., et al., *Macrophages as tools and targets in cancer therapy.* Nature Reviews Drug Discovery, 2022. **21**(11): p. 799-820.
19. Cuccarese, M.F., et al., *Heterogeneity of macrophage infiltration and therapeutic response in lung carcinoma revealed by 3D organ imaging.* Nat Commun, 2017. **8**: p. 14293.
20. Chevrier, S., et al., *An Immune Atlas of Clear Cell Renal Cell Carcinoma.* Cell, 2017. **169**(4): p. 736-749.e18.

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21. Laviron, M., et al., *Tumor-associated macrophage heterogeneity is driven by tissue territories in breast cancer.* Cell Rep, 2022. **39**(8): p. 110865.
22. Nalio Ramos, R., et al., *Tissue-resident FOLR2(+) macrophages associate with CD8(+) T cell infiltration in human breast cancer.* Cell, 2022. **185**(7): p. 1189-1207.e25.
23. Yano, S., et al., *Spatial-temporal FUCCI imaging of each cell in a tumor demonstrates locational dependence of cell cycle dynamics and chemoresponsiveness.* Cell Cycle, 2014. **13**(13): p. 2110-9.
24. Huang, Y.K., et al., *Macrophage spatial heterogeneity in gastric cancer defined by multiplex immunohistochemistry.* Nat Commun, 2019. **10**(1): p. 3928.
25. Lyssiotis, C.A. and A.C. Kimmelman, *Metabolic Interactions in the Tumor Microenvironment.* Trends Cell Biol, 2017. **27**(11): p. 863-875.
26. Raghunand, N., R.A. Gatenby, and R.J. Gillies, *Microenvironmental and cellular consequences of altered blood flow in tumours.* Br J Radiol, 2003. **76 Spec No 1**: p. S11-22.
27. Gillies, R.J., et al., *Targeting acidity in cancer and diabetes.* Biochim Biophys Acta Rev Cancer, 2019. **1871**(2): p. 273-280.
28. El-Kenawi, A., et al., *Acidity promotes tumour progression by altering macrophage phenotype in prostate cancer.* Br J Cancer, 2019. **121**(7): p. 556-566.
29. Bohn, T., et al., *Tumor immunoevasion via acidosis-dependent induction of regulatory tumor-associated macrophages.* Nat Immunol, 2018. **19**(12): p. 1319-1329.
30. Jiang, W., et al., *Extracellular Acidity Reprograms Macrophage Metabolism and Innate Responsiveness.* The Journal of Immunology, 2021. **206**(12): p. 3021-3031.
31. Partch, C.L., C.B. Green, and J.S. Takahashi, *Molecular architecture of the mammalian circadian clock.* Trends Cell Biol, 2014. **24**(2): p. 90-9.
32. Walton, Z.E., et al., *Acid Suspends the Circadian Clock in Hypoxia through Inhibition of mTOR.* Cell, 2018. **174**(1): p. 72-87.e32.
33. Mure, L.S., et al., *Diurnal transcriptome atlas of a primate across major neural and peripheral tissues.* Science, 2018. **359**(6381).
34. Zhang, R., et al., *A circadian gene expression atlas in mammals: implications for biology and medicine.* Proc Natl Acad Sci U S A, 2014. **111**(45): p. 16219-24.
35. Haspel, J.A., et al., *Perfect timing: circadian rhythms, sleep, and immunity - an NIH workshop summary.* JCI Insight, 2020. **5**(1).
36. Keller, M., et al., *A circadian clock in macrophages controls inflammatory immune responses.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21407-12.
37. Silver, A.C., et al., *Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells.* Brain Behav Immun, 2012. **26**(3): p. 407-13.
38. Nguyen, K.D., et al., *Circadian gene Bmal1 regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes.* Science, 2013. **341**(6153): p. 1483-8.
39. Baumann, A., et al., *The circadian clock is functional in eosinophils and mast cells.* Immunology, 2013. **140**(4): p. 465-74.
40. Adrover, J.M., et al., *A Neutrophil Timer Coordinates Immune Defense and Vascular Protection.* Immunity, 2019. **50**(2): p. 390-402.e10.
41. Arjona, A. and D.K. Sarkar, *Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells.* J Immunol, 2005. **174**(12): p. 7618-24.
42. Druzd, D., et al., *Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses.* Immunity, 2017. **46**(1): p. 120-132.
43. Scheiermann, C., et al., *Clocking in to immunity.* Nat Rev Immunol, 2018. **18**(7): p. 423-437.
44. Kitchen, G.B., et al., *The clock gene Bmal1 inhibits macrophage motility, phagocytosis, and impairs defense against pneumonia.* Proc Natl Acad Sci U S A, 2020.

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45. Gibbs, J.E., et al., *The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines.* Proc Natl Acad Sci U S A, 2012. **109**(2): p. 582-7.
46. He, W., et al., *Circadian Expression of Migratory Factors Establishes Lineage-Specific Signatures that Guide the Homing of Leukocyte Subsets to Tissues.* Immunity, 2018. **49**(6): p. 1175-1190.e7.
47. Nobis, C.C., et al., *The circadian clock of CD8 T cells modulates their early response to vaccination and the rhythmicity of related signaling pathways.* Proc Natl Acad Sci U S A, 2019. **116**(40): p. 20077-20086.
48. Fortier, E.E., et al., *Circadian variation of the response of T cells to antigen.* J Immunol, 2011. **187**(12): p. 6291-300.
49. Beam, C.A., et al., *Synchronization of the Normal Human Peripheral Immune System: A Comprehensive Circadian Systems Immunology Analysis.* Sci Rep, 2020. **10**(1): p. 672.
50. Mazzoccoli, G., et al., *A timetable of 24-hour patterns for human lymphocyte subpopulations.* J Biol Regul Homeost Agents, 2011. **25**(3): p. 387-95.
51. Silver, A.C., et al., *The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity.* Immunity, 2012. **36**(2): p. 251-61.
52. Phillips, A.C., et al., *Preliminary evidence that morning vaccination is associated with an enhanced antibody response in men.* Psychophysiology, 2008. **45**(4): p. 663-6.
53. Long, J.E., et al., *Morning vaccination enhances antibody response over afternoon vaccination: A cluster-randomised trial.* Vaccine, 2016. **34**(24): p. 2679-85.
54. Curtis, A.M., et al., *Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1.* Proc Natl Acad Sci U S A, 2015. **112**(23): p. 7231-6.
55. Deng, W., et al., *The Circadian Clock Controls Immune Checkpoint Pathway in Sepsis.* Cell Rep, 2018. **24**(2): p. 366-378.
56. Aiello, I., et al., *Circadian disruption promotes tumor-immune microenvironment remodeling favoring tumor cell proliferation.* Sci Adv, 2020. **6**(42).
57. Tsuruta, A., et al., *Diurnal Expression of PD-1 on Tumor-Associated Macrophages Underlies the Dosing Time-Dependent Antitumor Effects of the PD-1/PD-L1 Inhibitor BMS-1 in B16/BL6 Melanoma-Bearing Mice.* Mol Cancer Res, 2022. **20**(6): p. 972-982.
58. Strauss, L., et al., *Targeted deletion of PD-1 in myeloid cells induces antitumor immunity.* Sci Immunol, 2020. **5**(43).
59. Qian, D.C., et al., *Effect of immunotherapy time-of-day infusion on overall survival among patients with advanced melanoma in the USA (MEMOIR): a propensity score-matched analysis of a single-centre, longitudinal study.* Lancet Oncol, 2021. **22**(12): p. 1777-1786.
60. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
61. Yoo, S.H., et al., *PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5339-46.
62. Rodheim, K., C. Jung, and K. Wright, *016 Associations between Circadian Melatonin and Temperature Amplitudes during Constant Routine.* Sleep, 2021. **44**(Supplement_2): p. A8-A8.
63. Wu, G., et al., *Normalized coefficient of variation (nCV): a method to evaluate circadian clock robustness in population scale data.* Bioinformatics, 2021. **37**(23): p. 4581-4583.
64. Chen, S., et al., *A Pro- and Anti-inflammatory Axis Modulates the Macrophage Circadian Clock.* Front Immunol, 2020. **11**: p. 867.
65. Lellupitiyage Don, S.S., et al., *Macrophage circadian rhythms are differentially affected based on stimuli.* Integr Biol (Camb), 2022. **14**(3): p. 62-75.

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66. Pilorz, V., et al., *A Novel Mechanism Controlling Resetting Speed of the Circadian Clock to Environmental Stimuli.* Current Biology, 2014. **24**(7): p. 766-773.
67. Abe, M., et al., *Circadian rhythms in isolated brain regions.* J Neurosci, 2002. **22**(1): p. 350-6.
68. Gaspar, L.S., et al., *The importance of determining circadian parameters in pharmacological studies.* British Journal of Pharmacology, 2019. **176**(16): p. 2827-2847.
69. Boedtkjer, E. and S.F. Pedersen, *The Acidic Tumor Microenvironment as a Driver of Cancer.* Annual Review of Physiology, 2020. **82**(1): p. 103-126.
70. Gillies, R.J., Z. Liu, and Z. Bhujwalla, *31P-MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate.* Am J Physiol, 1994. **267**(1 Pt 1): p. C195-203.
71. Estrella, V., et al., *Acidity Generated by the Tumor Microenvironment Drives Local Invasion.* Cancer Research, 2013. **73**(5): p. 1524-1535.
72. Lv, S., et al., *A negative feedback loop of ICER and NF-κB regulates TLR signaling in innate immune responses.* Cell Death Differ, 2017. **24**(3): p. 492-499.
73. Kraut, J.A. and N.E. Madias, *Lactic acidosis.* N Engl J Med, 2014. **371**(24): p. 2309-19.
74. Pastorekova, S. and R.J. Gillies, *The role of carbonic anhydrase IX in cancer development: links to hypoxia, acidosis, and beyond.* Cancer Metastasis Rev, 2019. **38**(1-2): p. 65-77.
75. Swietach, P., *What is pH regulation, and why do cancer cells need it?* Cancer Metastasis Rev, 2019. **38**(1-2): p. 5-15.
76. de la Cruz-López, K.G., et al., *Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches.* Front Oncol, 2019. **9**: p. 1143.
77. Colegio, O.R., et al., *Functional polarization of tumour-associated macrophages by tumour-derived lactic acid.* Nature, 2014. **513**(7519): p. 559-63.
78. Polumuri, S., D.J. Perkins, and S.N. Vogel, *cAMP levels regulate macrophage alternative activation marker expression.* Innate Immun, 2021. **27**(2): p. 133-142.
79. Tavares, L.P., et al., *Blame the signaling: Role of cAMP for the resolution of inflammation.* Pharmacol Res, 2020. **159**: p. 105030.
80. Yagita, K. and H. Okamura, *Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts.* FEBS Lett, 2000. **465**(1): p. 79-82.
81. O'Neill, J.S., et al., *cAMP-dependent signaling as a core component of the mammalian circadian pacemaker.* Science, 2008. **320**(5878): p. 949-53.
82. Misra, U.K. and S.V. Pizzo, *Coordinate regulation of forskolin-induced cellular proliferation in macrophages by protein kinase A/cAMP-response element-binding protein (CREB) and Epac1-Rap1 signaling: effects of silencing CREB gene expression on Akt activation.* J Biol Chem, 2005. **280**(46): p. 38276-89.
83. Geeraerts, X., et al., *Macrophage Metabolism As Therapeutic Target for Cancer, Atherosclerosis, and Obesity.* Front Immunol, 2017. **8**: p. 289.
84. Shilts, J., G. Chen, and J.J. Hughey, *Evidence for widespread dysregulation of circadian clock progression in human cancer.* PeerJ, 2018. **6**: p. e4327.
85. Pariollaud, M., et al., *Circadian disruption enhances HSF1 signaling and tumorigenesis in Kras-driven lung cancer.* Science Advances, 2022. **8**(39): p. eabo1123.
86. Besmer, D.M., et al., *Pancreatic ductal adenocarcinoma mice lacking mucin 1 have a profound defect in tumor growth and metastasis.* Cancer Res, 2011. **71**(13): p. 4432-42.
87. Kidiyoor, A., et al., *Pancreatic Cancer Cells Isolated from Muc1-Null Tumors Favor the Generation of a Mature Less Suppressive MDSC Population.* Front Immunol, 2014. **5**: p. 67.
88. Alexander, R.K., et al., *Bmal1 integrates mitochondrial metabolism and macrophage activation.* eLife, 2020. **9**: p. e54090.
89. Lee, C.-C., et al., *Macrophage-secreted interleukin-35 regulates cancer cell plasticity to facilitate metastatic colonization.* Nature Communications, 2018. **9**(1): p. 3763.

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90. Walton, Z.E., R.C. Brooks, and C.V. Dang, *mTOR Senses Intracellular pH through Lysosome Dispersion from RHEB.* Bioessays, 2019: p. e1800265.
91. Guellaen, G., et al., *RMI 12330 A, an inhibitor of adenylate cyclase in rat liver.* Biochim Biophys Acta, 1977. **484**(2): p. 465-75.
92. Hunt, N.H. and T. Evans, *RMI 12330A, an inhibitor of cyclic nucleotide phosphodiesterases and adenylate cyclase in kidney preparations.* Biochim Biophys Acta, 1980. **613**(2): p. 499-506.
93. van Rossum, D.B., et al., *Ca2+ entry mediated by store depletion, S-nitrosylation, and TRP3 channels. Comparison of coupling and function.* J Biol Chem, 2000. **275**(37): p. 28562-8.
94. O'Neill, J.S. and A.B. Reddy, *The essential role of cAMP/Ca2+ signalling in mammalian circadian timekeeping.* Biochem Soc Trans, 2012. **40**(1): p. 44-50.
95. Ni, L., et al., *Identification and Function of Acid-sensing Ion Channels in RAW 264.7 Macrophage Cells.* Curr Med Sci, 2018. **38**(3): p. 436-442.
96. Selezneva, A., A.J. Gibb, and D. Willis, *The contribution of ion channels to shaping macrophage behaviour.* Front Pharmacol, 2022. **13**: p. 970234.
97. Putker, M., et al., *Mammalian Circadian Period, But Not Phase and Amplitude, Is Robust Against Redox and Metabolic Perturbations.* Antioxidants & Redox Signaling, 2017. **28**(7): p. 507-520.
98. Viola, A., et al., *The Metabolic Signature of Macrophage Responses.* Front Immunol, 2019. **10**: p. 1462.
99. Morris, E.L., et al., *Single-cell transcriptomics of suprachiasmatic nuclei reveal a Prokineticin-driven circadian network.* Embo j, 2021. **40**(20): p. e108614.
100. Ma, D., et al., *A transcriptomic taxonomy of Drosophila circadian neurons around the clock.* Elife, 2021. **10**.
101. Auerbach, B.J., G.A. FitzGerald, and M. Li, *Tempo: an unsupervised Bayesian algorithm for circadian phase inference in single-cell transcriptomics.* Nat Commun, 2022. **13**(1): p. 6580.
102. Finger, A.M., et al., *Intercellular coupling between peripheral circadian oscillators by TGF-β signaling.* Sci Adv, 2021. **7**(30).
103. Massagué, J., *TGFb in Cancer.* Cell, 2008. **134**(2): p. 215-230.
104. Geiger, S.S., et al., *Daily variation in macrophage phagocytosis is clock-independent and dispensable for cytokine production.* Immunology, 2019.
105. Ramanathan, C., et al., *Cell Type-Specific Functions of Period Genes Revealed by Novel Adipocyte and Hepatocyte Circadian Clock Models.* PLOS Genetics, 2014. **10**(4): p. e1004244.
106. Committee, N.R.C., *Guide for the Care and Use of Laboratory Animals*, in *Guide for the Care and Use of Laboratory Animals*. 2011, National Academies Press (US)
Copyright © 2011, National Academy of Sciences.: Washington (DC).
107. Clausen, B.E., et al., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic Res, 1999. **8**(4): p. 265-77.
108. Storch, K.F., et al., *Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information.* Cell, 2007. **130**(4): p. 730-741.
109. Trouplin, V., et al., *Bone marrow-derived macrophage production.* J Vis Exp, 2013(81): p. e50966.
110. Gonçalves, R. and D.M. Mosser, *The Isolation and Characterization of Murine Macrophages.* Current Protocols in Immunology, 2015. **111**(1): p. 14.1.1-14.1.16.
111. Ray, A. and B.N. Dittel, *Isolation of mouse peritoneal cavity cells.* J Vis Exp, 2010(35).
112. Liu, P.S., et al., *alpha-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming.* Nat Immunol, 2017. **18**(9): p. 985-994.
113. Huber, A.L., et al., *CRY2 and FBXL3 Cooperatively Degrade c-MYC.* Mol Cell, 2016. **64**(4): p. 774-789.
114. Tomayko, M.M. and C.P. Reynolds, *Determination of subcutaneous tumor size in athymic (nude) mice.* Cancer Chemother Pharmacol, 1989. **24**(3): p. 148-54.

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115. Patro, R., et al., *Salmon provides fast and bias-aware quantification of transcript expression.* Nat Methods, 2017. **14**(4): p. 417-419.
116. Soneson, C., M.I. Love, and M.D. Robinson, *Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences.* F1000Res, 2015. **4**: p. 1521.
117. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies.* Nucleic Acids Res, 2015. **43**(7): p. e47.
118. Dai, M., et al., *Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data.* Nucleic Acids Res, 2005. **33**(20): p. e175.

Fig 1

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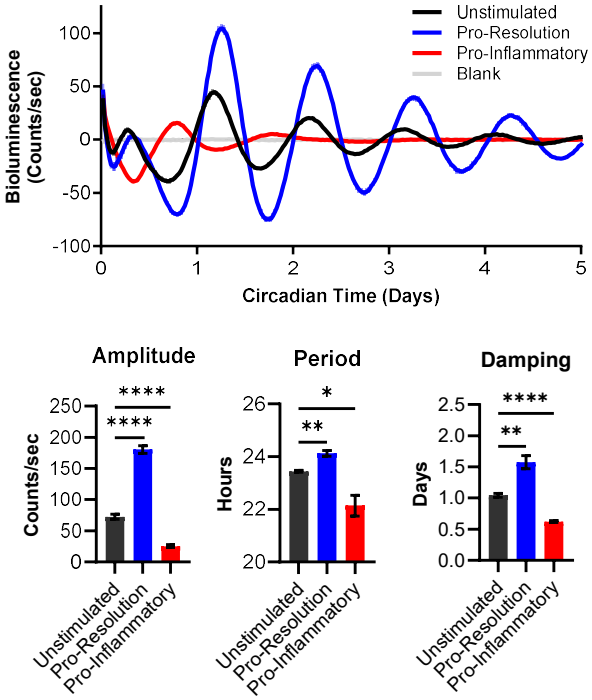

Fig 2

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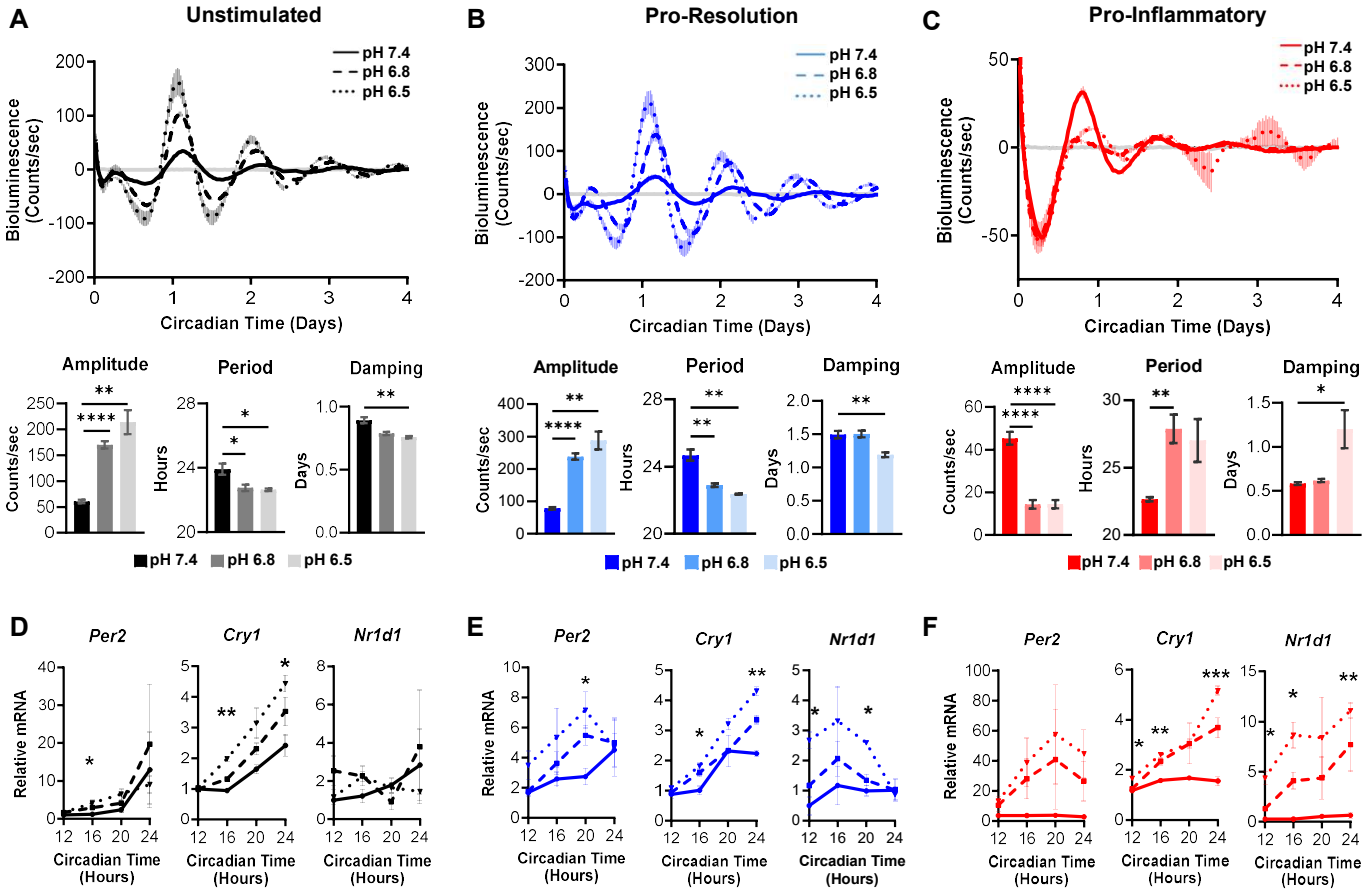

Fig 3

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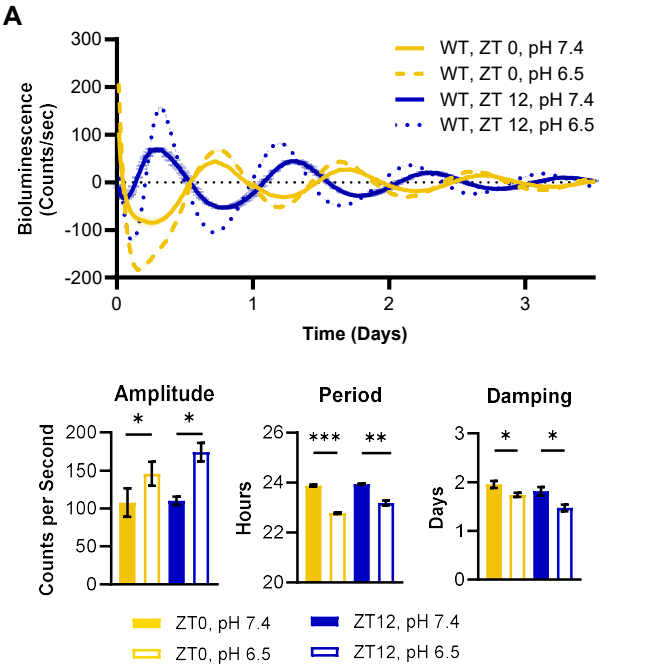

Fig 4

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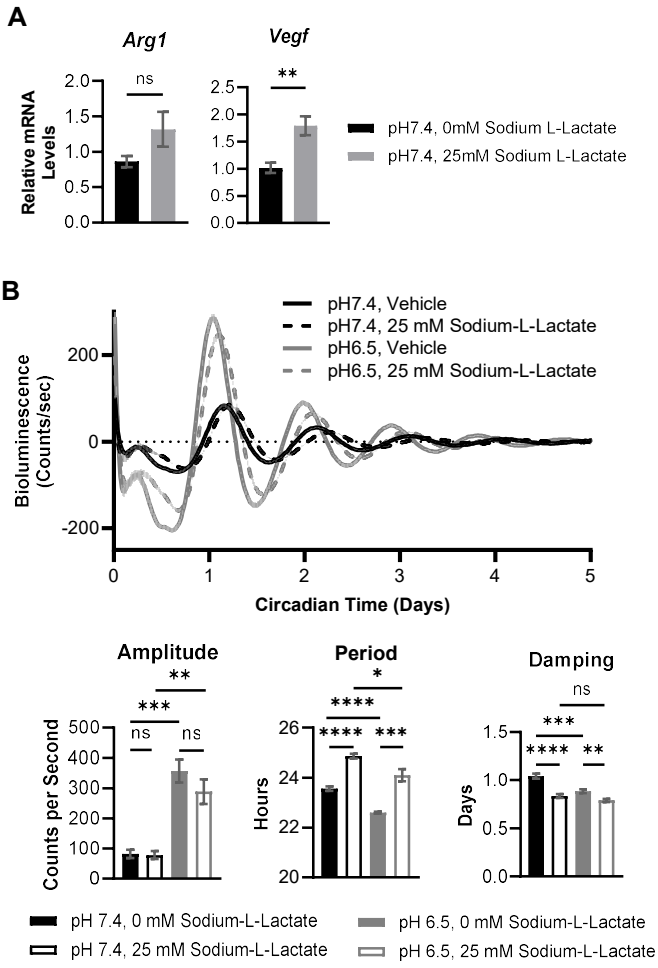

Fig 5

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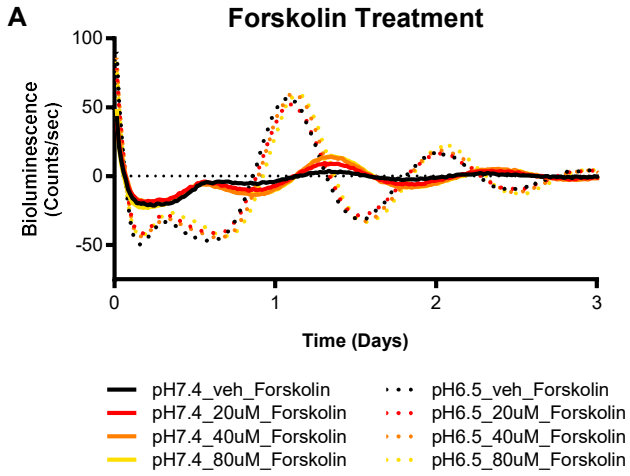

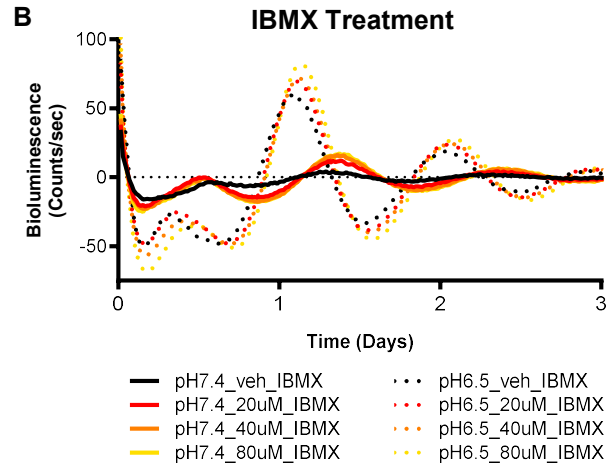

Fig 6

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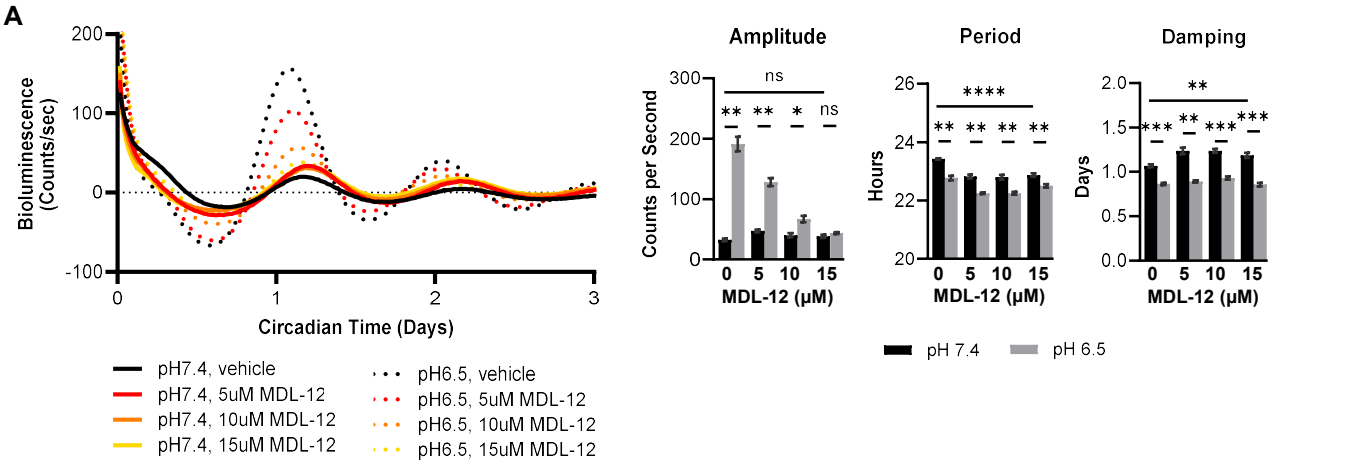

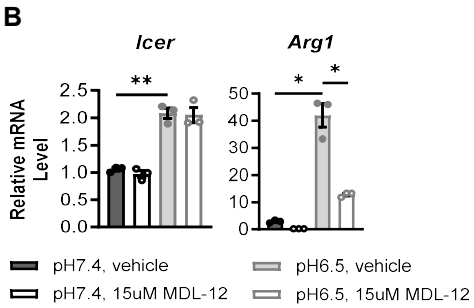

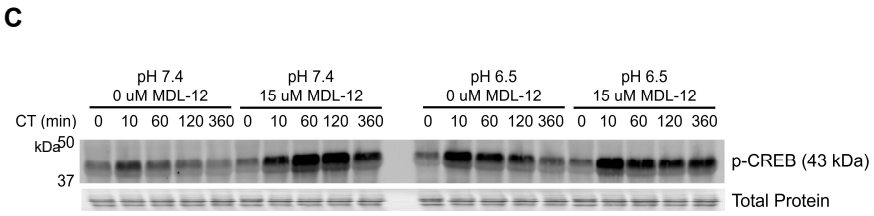

Fig 7

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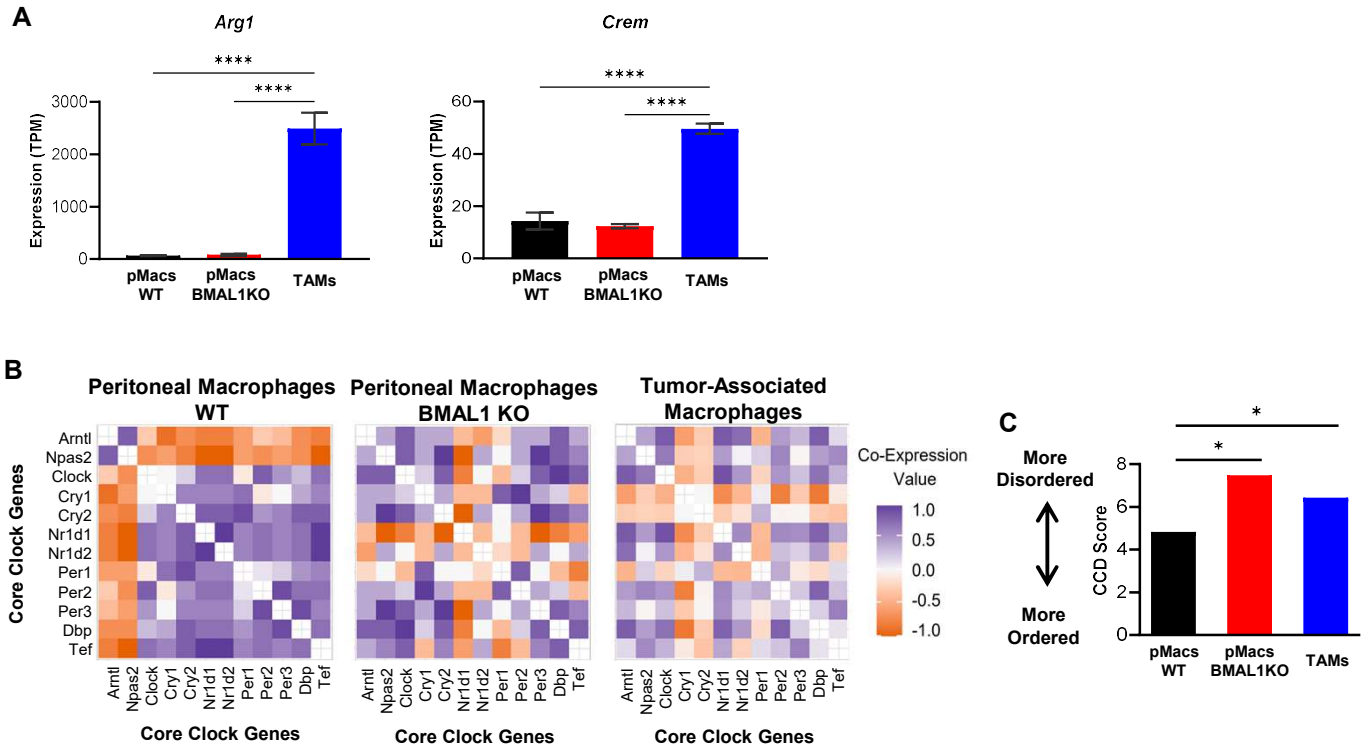

Fig 8

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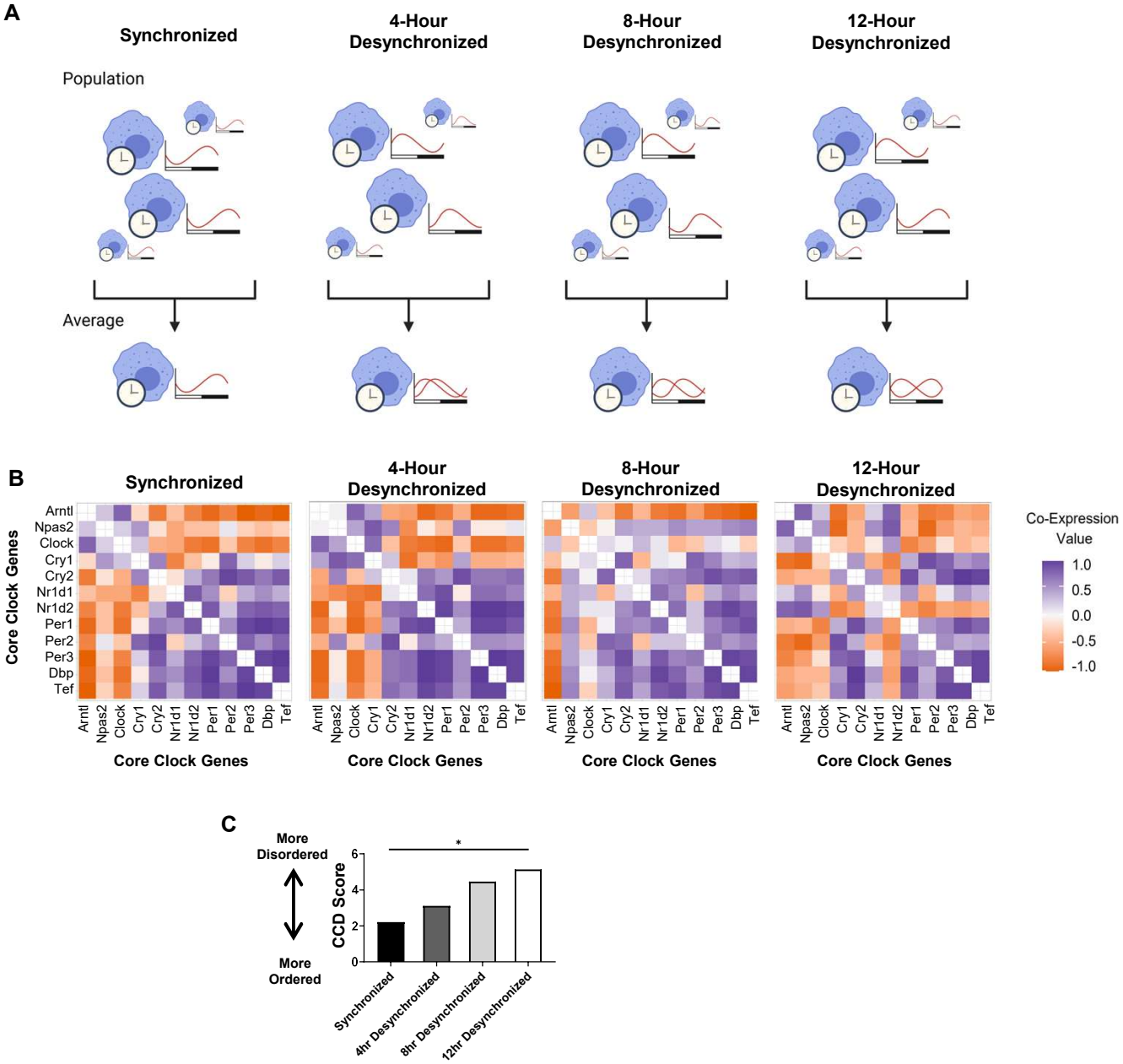

Fig 9

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