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1 **Title: Daytime variation in SARS-CoV-2 infection and cytokine production**

2 **Running title: Covid-19 and host circadian rhythm**

3

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26

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28 **Abstract**

29 S. Ray and A. Reddy recently anticipated the implication of circadian rhythm in severe acute
30 respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of the coronavirus
31 disease (Covid-19). In addition to its key role in the regulation of biological functions, the circadian
32 rhythm has been suggested as a regulator of viral infections. Specifically, the time of day of infection
33 was found critical for illness progression, as has been reported for influenza, respiratory syncytial
34 and parainfluenza type 3 viruses. We analyzed circadian rhythm implication in SARS-CoV-2 virus
35 infection of isolated human monocytes, key actor cells in Covid-19 disease, from healthy subjects.
36 The circadian gene expression of *BMAL1* and *CLOCK* genes was investigated with q-RT-PCR.
37 Monocytes were infected with SARS-CoV-2 virus strain and viral infection was investigated by
38 One-Step qRT-PCR and immunofluorescence. Interleukin (IL)-6, IL-1 β and IL-10 levels were also
39 measured in supernatants of infected monocytes. Using Cosinor analysis, we showed that *BMAL1*
40 and *CLOCK* transcripts exhibited circadian rhythm in monocytes with an acrophase and a
41 bathyphase at **Circadian Time (CT)6** and **CT17**. After forty-eight hours, the amount of SARS-CoV-2
42 virus increased in the monocyte infected at **CT6** compared to **CT17**. The high virus amount at **CT6**
43 was associated with significant increased release in IL-6, IL-1 β and IL-10 compared to **CT17**. Our
44 results suggest that time day of SARS-CoV-2 infection affects viral infection and host immune
45 response. They support consideration of circadian rhythm in SARS-CoV-2 disease progression and
46 we propose circadian rhythm as a novel target for managing viral progression.

47

48 **Key words:** Covid-19, circadian rhythm, monocytes, inflammatory cytokine

49 **Introduction**

50 The implication of circadian rhythm (CR) in pathogenesis of Severe Acute Respiratory
51 Syndrome Coronavirus 2 (SARS-CoV-2) has been recently anticipated (1). The CR regulates
52 physiological processes in living organisms with a period of 24 hours (2). Rhythmicity depends on
53 central and peripheral oscillators whose activity relies on two main feedback loops managed by a
54 clock genes cascade under the regulation of the main clock gene *BMALI* (2). The host susceptibility
55 to microorganism is likely under control of biological clocks (3). The time of day of infection is
56 critical for illness progression as reported for influenza, respiratory syncytial and parainfluenza type
57 3 viruses (4–6). We previously reported that CR is a key actor at the interface between infection
58 susceptibility, clinical presentation and prognosis of infection (3, 7).

59 There are some [evidence](#) that enable to anticipate the role of CR in SARS-CoV-2 infection.
60 The absence of *Bmal1* has an impact on intracellular replication of coronaviruses, especially
61 vesicular trafficking, endoplasmic reticulum and protein biosynthesis (8). Knock-out of *BMALI*
62 markedly decreases the replication of several viruses such as Dengue or Zika (9). Finally, among key
63 proteins involved in SARS-CoV-2 interaction with the host recently published (10), it has been
64 identified 30% of them being associated with circadian pathway (1). Clearly, [the evidence](#) of an
65 implication of CR in SARS-CoV-2 infection of human cells are lacking. In this study, we wondered
66 if SARS-CoV-2 infection and cytokine production by human monocytes, innate immune cells
67 affected by Covid-19, were regulated by CR.

68 **Results and Discussion**

69 We first investigated circadian oscillations of resting monocytes. Every 3 hours during 24
70 hours, total RNA was extracted and expression of *BMAL1* and *CLOCK* genes was investigated in
71 unstimulated monocytes. Expression of investigated genes exhibited CR in monocytes with an
72 acrophase (peak of the rhythm) and a bathyphase (trough of the rhythm) at Circadian Time (CT)6
73 and CT17, respectively (Fig.1A, Table 1). These two time points represent the beginning of the
74 active and the resting periods in humans (11). To assess the involvement of CR in infection of
75 monocytes with SARS-CoV-2, we incubated monocytes with SARS-CoV-2 at the bathyphase (CT6)
76 or at the acrophase (CT17) during 48 hours. As illustrated in the Fig.1B, viral uptake by monocytes
77 was higher at CT6 than at CT17. Additionally, we showed that the amounts of both SARS-CoV-2
78 RNA virus (Fig. 1C) and titer (Fig. 1D) were higher in supernatants of monocytes infected at CT6
79 than at CT17 (Fig.1B). Our data showed for the first time that entry and multiplication of SARS-
80 CoV-2 in human monocytes varies with the time of day. This finding is reminiscent of what has been
81 previously reported with herpes and influenza virus in murine models of infection (6). It is
82 noteworthy that CRs are different in rodents and humans, thus limiting extrapolations to
83 understand pathogenesis of SARS-CoV-2 infection.

84 Covid-19 disease is characterized by runaway immune system leading to a cytokine storm
85 consisting of high circulating levels of cytokines including IL-6, IL-1 β and IL-10 (12). We wondered
86 if the interaction of SARS-CoV-2 with monocytes affected cytokine production at two points of the
87 CR. The amounts of IL-6, IL-1 β and IL-10 were significantly increased at CT6 (Fig.1E) when the
88 amount of infection is highest. Hence, the interaction of SARS-CoV-2 with monocytes resulted in
89 distinct cytokine pattern according to daytime.

90 We demonstrate here that the time day of SARS-CoV-2 infection determines consistently
91 viral infection/replication and host immune response. It is likely that SARS-CoV-2 exploits clock
92 pathway for its own gain. Our findings support consideration of CR in SARS-CoV-2 disease
93 progression and suggest that CR represents a novel target for managing viral progression. This study
94 also highlights the importance of the time of treatment administration to Covid-19 patients since CR
95 was found regulating pharmacokinetics of several drugs (13). Several treatments are proposed to
96 prevent the occurrence of severe forms in Covid-19. They include passive immunization, cytokines,
97 anti-cytokine antibody or corticoids (14). All these candidates affect the immune response known to
98 oscillate during the day and their administration according to CR of SARS-CoV-2. Finally, the well-
99 documented CR disturbance in intensive care units (15) should be considered in the clinical and
100 therapeutic management of patients with severe Covid-19.

101 **Methods**

102 **Cells and virus**

103 SARS-CoV-2 strain MI6 was cultured in Vero E6 cells (American type culture collection ATCC®
104 CRL-1586™) in Minimum Essential Media (Life Technologies, Carlsbad, CA, USA) supplemented
105 with 4% fetal bovine serum (FBS). The viral titer was evaluated using the TCID50 method
106 corresponding to the amount of virus inducing a cytopathic effect in 50% of infected Vero E6 cells.
107 Blood from anonymous healthy donors (n=4, 1 men (38 year) and 3 women (28, 34 and 39 year)
108 were collected from leukopacks (convention n°7828, Etablissement Français du Sang, Marseille,
109 France) at one point and peripheral blood mononuclear cells (PBMCs) were retrieved and frozen. At
110 the beginning of the experiment, human monocytes were isolated from PBMCs of each donors
111 following CD14 selection using MACS magnetic beads (Miltenyi Biotec, Bergisch, Germany) as
112 previously described (16). The purity of CD14 cells after selection was 98%. Monocytes ($5 \cdot 10^5$
113 cells/well) were cultured in Roswell Park Memorial Institute medium-1640 (Life Technologies)
114 containing 10% of FBS, 100 U/mL penicillin and 50 µg/mL streptomycin (Life Technologies).
115 After isolation, the cells were cultured for 12 hours and then were infected with 50 µl virus
116 suspension (0.1 multiplicity of infection (MOI)) at the bathyphase (CT6) and acrophase (CT17) for
117 48 hours at 37°C in the presence of 5% CO₂.

118

119 **Circadian gene expression**

120 Isolated monocytes were cultured for 12 hours and then the circadian rhythm was assessed from
121 midnight every 3 hours for 24 hours. Total RNA was extracted using the RNA Mini Kit (Qiagen)
122 and a quantitative Real-Time PCR was performed according to the manufacturer's instructions
123 (MMLV Kit, Life Technologies and Smart SYBRGreen kit, Roche Applied Science). Circadian gene
124 expression was investigated using specific primers targeting *BMAL1* (5'-
125 3'AAACCAACTTTTCTATCAGACGATGAA; 3'-5' TCGGTCACATCCTACGACAAAC) and
126 *CLOCK* (5'-3' AAGTTAGGGCTGAAAGACGACG; 3'-5' GAACTCCGAGAAGAGGCAGAAG)
127 genes (7). Results were normalized using *ACTB* gene (β-actin) (5'-3'
128 GGAAATCGTGCGTGACATTA; 3'-5' AGGAAGGAAGGCTGGAAGAG) as it was previously
129 observed that this housekeeping endogenous gene did not oscillate over time (17, 18). The results are
130 expressed according to the appropriate formula: gene expression = $\text{Log}(2^{-\Delta\text{Ct}})$ relative expression,
131 with Ct (Cycle threshold), $\Delta\text{Ct} = \text{Ct target gene} - \text{Ct } \beta\text{-actin}$. The Cosinor analysis based on an
132 extrapolation from measurements of a few points over 24 hours was used to evaluate the CR of the
133 clock genes *BMAL1* and *CLOCK*.

134

135 **Viral RNA extraction and PCR**

136 Viral RNA was extracted from the [supernatant of infected cells](#) using NucleoSpin® Viral RNA
137 Isolation kit (Macherey-Nagel) and Covid-19 virus detection was performed using One-Step qRT-
138 PCR SuperScript™ III Platinum™ (Life Technologies) targeting the gene E.

139

140 **Immunofluorescence**

141 Infected cells ($5 \cdot 10^5$ cells/well) were fixed and incubated with phalloidin-555 and 4',6-diamidino-2-
142 phenylindole (DAPI) to label F-actin and nucleus respectively. SARS-CoV-2 virus was labelled
143 using first an anti-SARS-CoV-2 antibody (Spike protein, Thermo [Fisher](#)) and then a secondary anti-
144 rabbit Alexa 647 (Thermo Fisher).

145 Pictures were acquired using confocal microscopy (LSM 8000 Airyscan confocal microscope, x 63,
146 oil objective) [and the phagocytosis activity of monocytes was determined as the phagocytosis index](#)
147 [according to the following formula \(evaluating 100 cells for each donor\)](#): percentage of phagocytosis
148 $((\text{average number of infected cells} \times 100) / \text{total number of counted cells}) \times \text{average number of}$
149 $\text{particles or viruses/cells}$.

150

151 **Immunoassays**

152 Levels of interleukin (IL)-6, IL-1 β and IL-10 were measured in cell supernatants using an enzyme-
153 linked immunosorbent assay technique (R&D systems). The sensitivity of the assays was (pg/ml)
154 15.4 for IL-6, 0.125 for IL-1 β and 3.9 for IL-10.

155

156 **Statistical analysis**

157 Statistical analyses were performed with GraphPad Prism (7.0, La Jolla, CA) and R studio v3.4.0.
158 Continuous variables were expressed as medians \pm interquartile, and comparisons between two
159 groups were made using the Mann-Whitney non-parametric test for unmatched data and the Student
160 t-test for matched data.

161 [For CR analysis, we used the Cosinor transformation to estimate for a given variable its variations](#)
162 [over a 24-hour period. CR parameters were investigated including the acrophase \(time elapsed until a](#)
163 [maximum activity\) and its inverse the batyphase, the amplitude \(half of the maximum variation of](#)
164 [the considered rhythm\) and the mesor \(average gene expression\). A significant CR is defined when](#)
165 [the three circadian parameters are statistically significant. Statistical significance was defined as](#)
166 $P \leq 0.05$.

167

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174

175 **Disclosure Statement**

176 The authors declare no conflict of interest.

177

178 **Author contributions**

179 A.B.D and L.G performed experiments. A.B.D, L.G, B.C and S.M analyzed the data. A.B.D, M.L,
180 S.M and J.L.M supervised the work. A.B.D, S.M and J.L.M wrote the manuscript. All authors
181 reviewed and approved the submitted manuscript. All authors reviewed the draft of the
182 manuscript and provided intellectual input.

183

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247

248 **Figure legends**

249 **Figure 1. SARS-CoV-2 infection is link to circadian rhythm**

250 (A) Circadian rhythm of *BMAL1* and *CLOCK* genes in monocytes using Cosinor model from 4
251 different donors. (B) Virus load (n=4) and titers (n=3) at CT6 and CT17 time from experiments that
252 were repeated 3 times using at least 3 different donors. (C) Representative pictures and phagocytosis
253 activity of monocytes (F-actin in green and nucleus in blue) infected by SARS-CoV-2 virus (red).
254 Results are expressed as the mean \pm SEM of 4 donors with 3 independent experiments. (D) Level of
255 IL-6, IL-1 β and IL-10 of unstimulated (red) and infected cells at CT6 and CT17. Results are
256 expressed as the mean of 4 donors from 3 independent experiments.

257

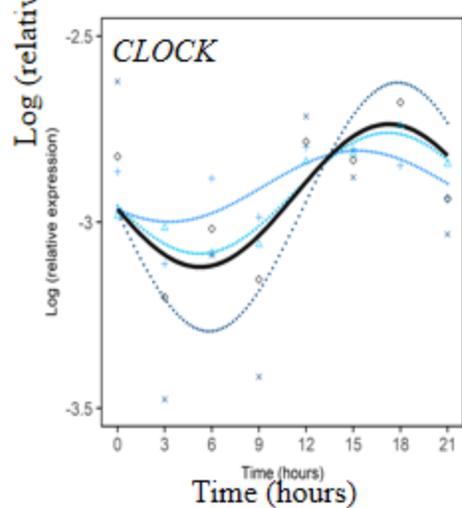
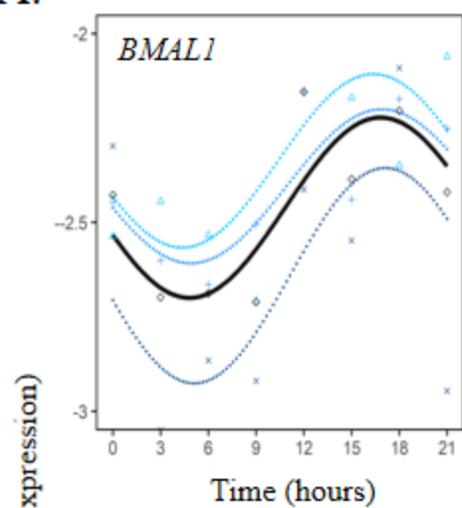
258 **Table.**

259 **Table 1. Rhythmic parameters (mesor, amplitude and acrophase) for *BMAL1* and *CLOCK***
260 **genes**

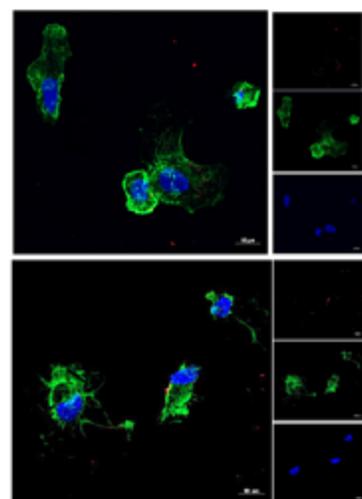
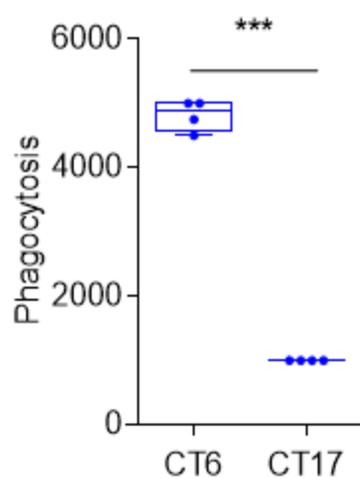
Gene	Donors	Mesor	<i>P</i> -value	Amplitude	<i>P</i> -value	Acrophase	<i>P</i> -value
<i>BMAL1</i>	1	-2.33	<0.001	0.23	0.05	16.37	0.03
	2	-2.40	<0.001	0.20	0.003	16.91	<0.001
	3	-2.64	<0.001	0.28	0.008	17.12	0.02
<i>CLOCK</i>	1	-2.92	<0.001	0.16	<0.001	17.20	<0.001
	2	-2.90	<0.001	0.09	0.03	15.29	0.007
	3	-2.96	<0.001	0.33	0.03	17.83	0.001

261

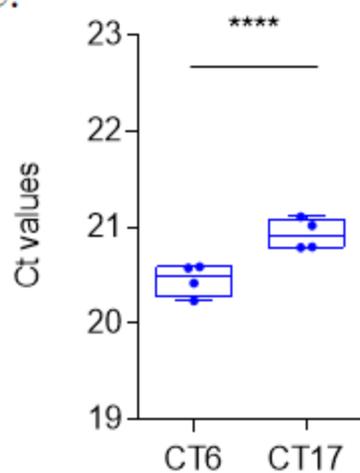
A.



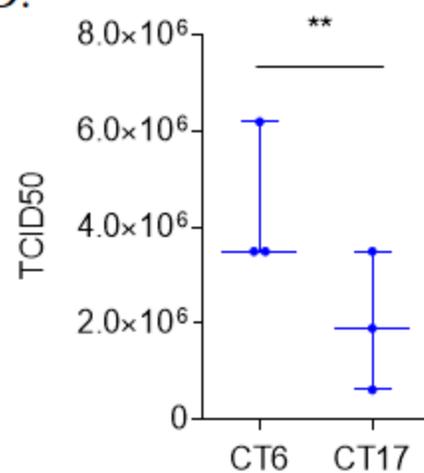
B.



C.



D.



E.

