UDC 571.27 https://doi.org/10.15407/biotech15.04.008

CIRCADIAN VARIATION IN FUNCTIONAL POLARIZATION OF MURINE PERITONEAL MACROPHAGES

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Received 01.07.2022
Revised 25.07.2022
Accepted 31.08.2022

Aim. This study aimed to investigate the circadian rhythm of the murine peritoneal macrophage (PM) metabolic profile.

Methods. The metabolic profile of PM was characterized by phagocytic activity, reactive oxygen species (ROS) generation, and by the expression of phenotypic markers, associated with a pro- and anti-inflammatory metabolic shift. Phagocytosis of FITC-labeled inactivated *Staphylococcus aureus*, ROS generation, CD80, CD86, and CD206 expression were estimated by flow cytometry at a regular 4h interval over the daily light-dark cycle.

Results. The phagocytic index and percentage of ROS-producing PM were found to be lower in the resting phase (ZT4) as compared to the active phase. In contrast, the level of CD86 expression was the highest in the inactive phase (ZT8). There was also a statistically significant peak in the proportion of ROS-producing PM, as well as in the level of ROS production per cell at the time of awakening (ZT12). As opposed to ROS generation, ZT12 was characterized by the lowest level of cell-surface CD206 expression.

Conclusions. Our results indicate that there is a circadian rhythm in functional polarization of murine PM with an anti-inflammatory activation state in the resting phase in comparison to the active phase.

Key words: circadian rhythm, peritoneal macrophages, phagocytosis, reactive oxygen species, phenotypic polarization.

It is known that circadian disruption plays a prominent role in the pathogenesis of many inflammatory disorders, such as obesity, diabetes mellitus, and others [1]. Peritoneal macrophages (PM) are important components of omentum-associated lymphoid tissue, and their involvement in the pathogenesis of different inflammatory diseases makes them a promising target for immunotherapy [2]. Despite a lot of research that demonstrated circadian rhythmicity of macrophage function [3], temporal variation in functional and phenotypic polarization of those cells remains understudied.

This study aimed to investigate the circadian rhythm in phagocytic activity, ROS production, and phenotype marker expression in murine PM.

Material and Methods

Male outbred mice were aged between 8–12 weeks. They were maintained on a 12 h light/12 h dark cycle (light: zeitgeber time (ZT) 0–12; dark: ZT 12–24). Murine PM were collected at 6 time points: ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24. Phagocytosis of FITC-labeled inactivated *Staphylococcus aureus*, ROS generation, CD80, CD86, and CD206 expression were estimated by flow cytometry. Data statistical significance was determined using one-way ANOVA followed by the post-hoc Tukey-Kramer HSD test. The values of $P < 0.05$ were considered significant.

Results and Discussion

We have not detected circadian rhythm in percentages of $CD206^+$, $CD80^+$, $CD86^+$, and phagocytic PM (data not shown). Rhythmic patterns in explored metabolic characteristics of PM varied significantly. The phagocytic index value of PM was found to be significantly lower in the resting phase (at ZT4) as compared to ZT12 (Fig. *A*). Phagocytic activity peaked at ZT12, at the beginning of the dark phase, followed by a progressive decrease until the pre-dawn time.

There was an increase in the proportion of ROS-producing PM at ZT12 in comparison to time points representing the resting phase (Post-Hoc Tukey HSD: ZT4, $P = 0.035$; ZT8, $P = 0.094$) and ZT16 (Post-Hoc Tukey HSD: $P = 0.06$). Also, a trend for the elevated percentage of ROS-generating PM at ZT20 (Post-Hoc Tukey HSD: $P = 0.048$) and ZT24 (Post-Hoc Tukey HSD: $P = 0.091$) was observed as compared to ZT4 (data not shown).

Likewise, there was a pronounced spike in the level of ROS production per cell at ZT12 in comparison to ZT4, ZT8, ZT16, and ZT24 (Fig. *B*). High ROS generation is the feature of pro-inflammatory (M1) macrophages. ZT12 marks the start of the dark period, and thus, the beginning of the active phase of mice as nocturnal animals. There is a lot of data showing that the concentration of many proinflammatory cytokines and chemokines increases near the onset of organism activity [4], which corroborates our finding about the peak ROS production, and therefore, the proinflammatory metabolic skew of murine PM at ZT12. Conversely, a lower percentage of ROS-producing PM was observed at ZT4 and ZT8, indicating that PM are shifted into an anti-inflammatory state during the inactive phase, and perform regenerative functions.

As opposed to ROS generation, ZT12 was characterized by the lowest level of CD206 expression in PM (Fig. *C*). There is not enough data in the literature about a diurnal variation

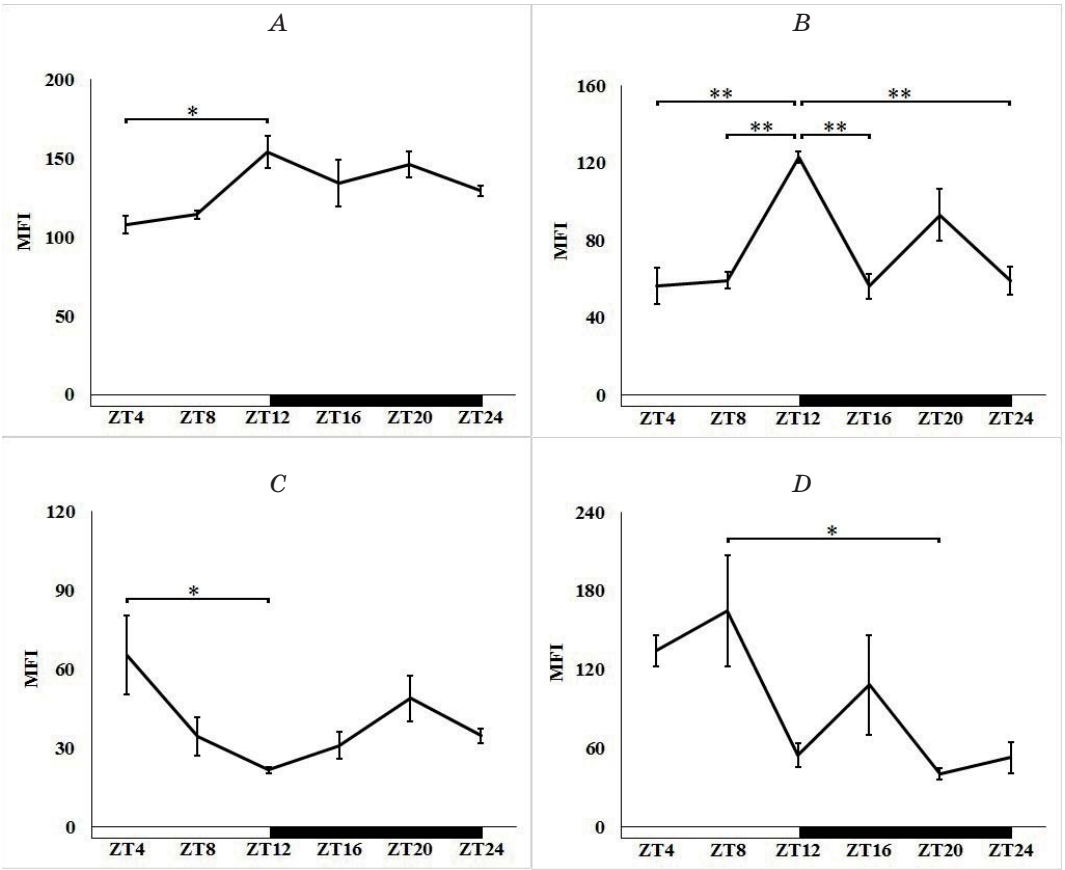

Fig. **Circadian variability in a functional state and phenotypic polarization of murine peritoneal macrophages:**

A — phagocytic index, *B* — level of ROS-production, *C* — CD206 expression level, *D* — CD86 expression level. MFI – mean fluorescence intensity.

$n = 3$ mice per time point. The data are presented as mean ± standard error of the mean.

$^*P < 0.05$; $^{**}P < 0.01$.

of CD206 expression in PMs. Kiessling et al. [5] discovered a higher percentage of $CD206^+$ PM in C57BL/6 J mice at CT9-CT15. The absence of such an effect in our experiment may be explained by the use of outbred mice in our study, which are characterized by higher genetic variability than inbred mice. Lower expression of CD206 per cell at ZT12 coincides with our data on ROS production described above, since CD206 is a marker of anti-inflammatory macrophages, and a decrease in its expression indicates pro-inflammatory phenotypic polarization of those cells.

There was no rhythmicity in the level of expression of CD80 on the PM cell surface at different time points. This observation coincides with the data obtained by Silver et al. [6], who found no significant difference in mRNA levels of this molecule in mice over the daily light-dark cycle. Conversely, the expression level of CD86 was higher in the resting phase (ZT8) in comparison to the active phase (Post-Hoc Tukey HSD: ZT12, $P = 0.069$; ZT20, $P = 0.035$; ZT24, $P = 0.063$) (Fig. *D*). In contrast to CD80, which is considered a marker of M1 macrophages, CD86 is used as a marker of both M1 and M2b macrophages [7]. Therefore, increased expression of CD86 in the resting phase in parallel with the absence of changes in the level of CD80 confirms our data regarding the anti-inflammatory phenotypic polarization of PM during that period since M2b is a subpopulation of alternatively activated macrophages with anti-inflammatory and immunoregulatory properties.

Conclusions

Our results indicated that there was a circadian rhythm in functional and phenotypic polarization of murine PM with an anti-inflammatory activation state in the resting phase as compared to the active phase, and a sharp proinflammatory shift of PM at the time of awakening (ZT12). This may be necessary for proper reparation of damaged tissues during sleep and fight against microorganisms amid activity when the risk of infection is more relevant.

Funding. The study was supported by a project funded by the Ministry of Education and Science of Ukraine (State Registration No.0120U102130).

Conflicts of interest
Authors declare no conflict of interest.

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