KEEPING THE RHYTHM: THE IMPACT OF CIRCADIAN CLOCK ON THE IMMUNE RESPONSE TO CANCER

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Abstract

The circadian clock, responsible for the regulation of physiological and behavioral functions in the body, has been found to play a significant role in the development of cancer. Studies have shown that circadian disruption due to atypical activity periods or shift work may lead to several types of cancer, including breast, prostate, and colorectal cancer. The development of cancer and its response to therapeutic protocols are strongly influenced by innate and adaptive immune systems. Indeed, the balance between the activation of inflammatory pathways and immunological tolerance is crucial for tumor development. Macrophages, which play a determinant role in the immune response to the tumor, can differentiate into pro-inflammatory (M1) or anti-inflammatory (M2) profiles depending on the signals they receive. In this work, we try to unveil how the circadian clock plays a significant part in the regulation of tumor immunity, focusing mainly on macrophages present in the tumor. Daily patterns in the presence and function of several leukocytes, including macrophages and dendritic cells, have been observed in the tumor context. In addition, the relation between the major profiles of macrophages, M1 and M2, is compromised under conditions that disrupt the circadian clock, favoring the immunological tolerance in the tumor microenvironment and, thus, helping the tumor to escape immune surveillance. On the other hand, the presence of the tumor itself affects the circadian system, which could initiate a vicious circle in which the circadian clock gets worse along with tumor progression, affecting the macrophages present in the tumor, among other leukocytes, favoring a more tolerogenic immunity within the tumor.

Keywords: Circadian System, Cancer, Tumor microenvironment, Immune system.

Resumen

El reloj circadiano, responsable de la regulación de las funciones fisiológicas y conductuales del cuerpo, ha demostrado desempeñar un papel significativo en el desarrollo del cáncer. Diferentes estudios han reportado que la disrupción circadiana debido a períodos de actividad atípicos o trabajo en turnos rotativos se asocia al establecimiento de diferentes tipos de cáncer, incluyendo el cáncer de mama, próstata y colorrectal. El desarrollo del cáncer y su respuesta a protocolos terapéuticos están fuertemente influenciados por el sistema inmune, tanto por la respuesta innata como adaptativa. El equilibrio entre la activación de las vías inflamatorias y la tolerancia inmunológica es crucial para la inmunidad tumoral. Los macrófagos, que juegan un papel crucial en la respuesta inmunológica en el microambiente tumoral, son capaces de diferenciarse en los perfiles pro-inflamatorios (M1) o anti-inflamatorios (M2) dependiendo de las señales que reciben. En este trabajo intentamos dilucidar cómo el reloj circadiano tiene un papel crucial en la inmunidad tumoral, centrándonos principalmente en los macrófagos presentes en el tumor. En el contexto tumoral, se han observado patrones diarios en la presencia y función de diferentes leucocitos, como macrófagos y células dendríticas. Adicionalmente, la relación entre los principales perfiles de macrófagos, M1 y M2, se ve comprometida en condiciones que alteran el funcionamiento del reloj circadiano, favoreciendo la inducción de tolerancia inmunológica en el microambiente tumoral y, por lo tanto, el escape del tumor a la vigilancia inmunológica. Por otro lado, la misma presencia del tumor afecta el funcionamiento del sistema circadiano, lo cual podría iniciar un círculo vicioso en el cual la desregulación circadiana afecta a los macrófagos presentes en el tumor, entre otros leucocitos, favoreciendo el desarrollo de tolerancia inmunológica.

Palabras clave: Sistema Circadiano, Cáncer, Microambiente Tumoral, Sistema inmune.

Received: May 11, 2023. Accepted in the final form: June 4, 2023

Introduction

The circadian clock is responsible for the generation and entrainment of daily rhythms for almost all physiological and behavioral functions of the body (*e.g.*, body temperature, hormonal secretion, sleep and locomotor activity), enabling adaptation to cyclic environmental changes. The molecular mechanism of the circadian clock arises from negative transcriptional feedback, which generates oscillations with periods close to 24 hours. In mammals, the core loop includes the positive elements Clock and Bmal1, which induce the expression of the negative elements Per1-3 and Cry1-2, which, in turn, repress the transcriptional activity of the positive elements. Along this process, compartmentalization, shuttling, and posttranslational events determine the pace of the circadian clock. This cell-autonomous pacemaker mechanism has been found in almost every cell in the body. The main biological clock resides in the hypothalamic suprachiasmatic nuclei (SCN) and the principal environmental signal that adjusts its activity is the light-dark (LD) cycle. In turn, the SCN control circadian oscillators in peripheral tissues (*e.g.*, liver, lung, and other brain areas) through neural, hormonal and behavioral pathways, to maintain an optimal phase relation between them and with the environment [1].

In mammals, shifting the activity period to an atypical time of the day (*e.g.*, by shift-work or jet-lag in humans) causes a temporal misalignment that, under chronic conditions, might result in disease onset (*e.g.*, cardio metabolic syndromes, obesity, cancer) and physical and mental health disorders. Epidemiological studies have shown that circadian disruption can affect the risk of developing several types of cancer, such as breast, prostate, and colorectal cancer [2]. It should be noted that the World Health Organization (WHO) has declared that shift work is a relevant risk factor for the development of cancer. In addition, circadian disruption can affect the effectiveness of cancer treatments, such as chemotherapy and radiation therapy [3].

The use of animal models has been pivotal to uncovering the genetic and environmental links that, when compromised by circadian disruption, lead to disease onset. A case in point was the initial finding that tumor-bearing mice increased their tumor growth rate when exposed to circadian desynchronization as experimental Chronic Jet-Lag (CJL), constant light (LL), or alterations of the molecular circadian clock [4]. Genome-wide transcript profiling showed that ~10% of the cell transcriptome, including genes involved in cell-cycle transition, response to DNA-damage, and cell death processes, were under circadian control [5]; all of which suggest a relevant role for clock components in establishing proliferative disorders.

The development of cancer and its response to therapeutic protocols are strongly influenced by innate and adaptive immune systems, which either promote or attenuate tumorigenesis. Chronic inflammation can promote tumor development and progression; nevertheless, the balance between the activation of immunological tolerance and inflammatory pathways is certainly relevant to tumor immunity. Tumor-specific or tumor-associated antigens can activate anti-tumor immune responses by dendritic cells (DC) and M1 macrophage-mediated activation of cytotoxic T-lymphocytes ($CD8^+$) and natural killer (NK) cells, among other cells. Additionally, induction of tolerance can be achieved by differentiation of immunosuppressive cells such as M2 macrophages, myeloid-derived suppressor cells (MDSC), tumor-associated neutrophils (TAN) and regulatory T cells (LTreg) [6]. Particularly, tumor associated macrophages (TAM), can differentiate into two functional profiles, M1 and M2 depending on the signal that they receive. Indeed, the M1 phenotype is more closely associated with inflammatory anti-tumor immunity whereas the M2 phenotype, a more functionally diverse subset of macrophages, exhibits immunosuppressive and homeostatic functions [7]. Moreover, the M1/M2 ratio has been associated with cancer prognosis [8]. As the circadian clock modulates several immune parameters, including the number of different cell types (*e.g.*, macrophages, $CD4^+$ and $CD8^+$ T cells) in different tissues (*e.g.*, peripheral blood, spleen), cytokine levels, phagocytic activity, and response to the immune challenge (*e.g.*, LPS) [reviewed in [9]], its derangement deregulates the inflammatory processes that are predicted to favor establishment and progression of tumors [10].

Taking these evidences into account, it is relevant to study circadian rhythms that govern the immune system associated with tumor development. The knowledge of the circadian regulation of the immune system in the context of cancer could be an interesting tool not only to improve current immune-mediated therapies, but also to design strategies to strengthen the circadian rhythm in order to increase the immune response against tumor cells.

Immune-mediated circadian regulation of tumor development

Although there is a lot of evidence about immune regulation of tumor growth, only a few studies take a circadian perspective. Recent data suggest that dysregulated circadian clock gene expression can enhance glioma progression by affecting the tumor immune response. Database analyses showed a strong association between the alteration of the clock-related genes and the enrichment of immunosuppressive cells, including Th2 cells, apoptotic and immature DC, in worst-prognosis glioma patients [11]. In glioblastoma patients, CLOCK seems to induce an immunosuppressive tumor microenvironment (TME) via upregulation of OLFML3, a novel and potent chemoattractant of immune suppressive microglia [12].

A recent work has shown that the time of day of tumor cells inoculation modulates the tumor growth rate, showing an increased rate at the end of the night in a murine melanoma model. Specifically, the rhythm in the traffic of DC to the tumor-draining lymph node, with maximum levels at the end of the day, governs a circadian response of tumor antigen-specific $CD8^+$ T cells that is dependent on the circadian expression of the co-stimulatory molecule CD80. As a consequence, cancer immunotherapy is more effective when synchronized with DC functions [13]. On the other hand, in rats carrying tumors, NK cells from the spleen showed higher nocturnal levels of Granzyme B, Perforin and Interferon (INF)-γ, circadian patterns that were lost under circadian desynchronization (CJL) [10]. In the murine melanoma, the population of M1 macrophages inside the tumor was increased during the night, while M2 macrophages were higher during the day, generating a M1/M2 ratio increased at night, daily patterns that, again, were lost under CJL. In addition, these mice showed increased levels of IL-1β, IL-6 and TNF-α during the day under LD condition but not under CJL [14]. Similarly, changes in the immune microenvironment inside the tumor, particularly an increase of tumor-supporting MHC II^{low} TAM and $CD4^+FoxP3^+$ Treg populations, together with a decrease in the number of infiltrating $CD8^+$ T cells, have been reported in a murine model of breast cancer subject to circadian deregulation [15]. Additionally, higher levels of leukocytes (total $CD45^+$ and LT $CD4^+$ cells) were observed in the tumors of animals subjected to CJL using a melanoma murine model [14]. Even though there are still few studies in this area, these data seem to suggest that the innate immune response increases at night, while adaptive immune response is higher during the day (Figure 1). In addition, circadian desynchronization not only disrupts circadian rhythms in immune cells but also increases immunosuppressive populations (See below).

Over the last years, immunotherapies have become a major treatment in cancer treatment, including the Immune checkpoint blockade (ICB) which consists in blocking checkpoint proteins from binding with their partner proteins [16]. One of the more used treatments recently is the ICB against the inhibitory receptors programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), since together with tumor progression there is an increase in the frequency of $PD1^+$ macrophages in the TME [17]. The interaction of PD1 with PD-L1, present in the lymphocytes and NK cells, inhibits the anti-tumoral functions of these cells. Results from a pilot study in patients with metastatic non-small-cell lung cancer has shown a time-dependent efficacy of Nivolumab, a monoclonal antibody against PD-1: morning dosing nearly quadrupled median progression-free and overall survival as compared to afternoon dosing [18].

These data demonstrate that the circadian rhythms of anti-tumor immune components are not only critical for controlling tumor growth but can also be of therapeutic relevance. In addition, the immune response includes both what happens inside the tumor, strongly dependent on TME, and the anti-tumor immune response developed in the secondary lymphatic organs along the body, which could be regulated by the circadian system through different pathways.

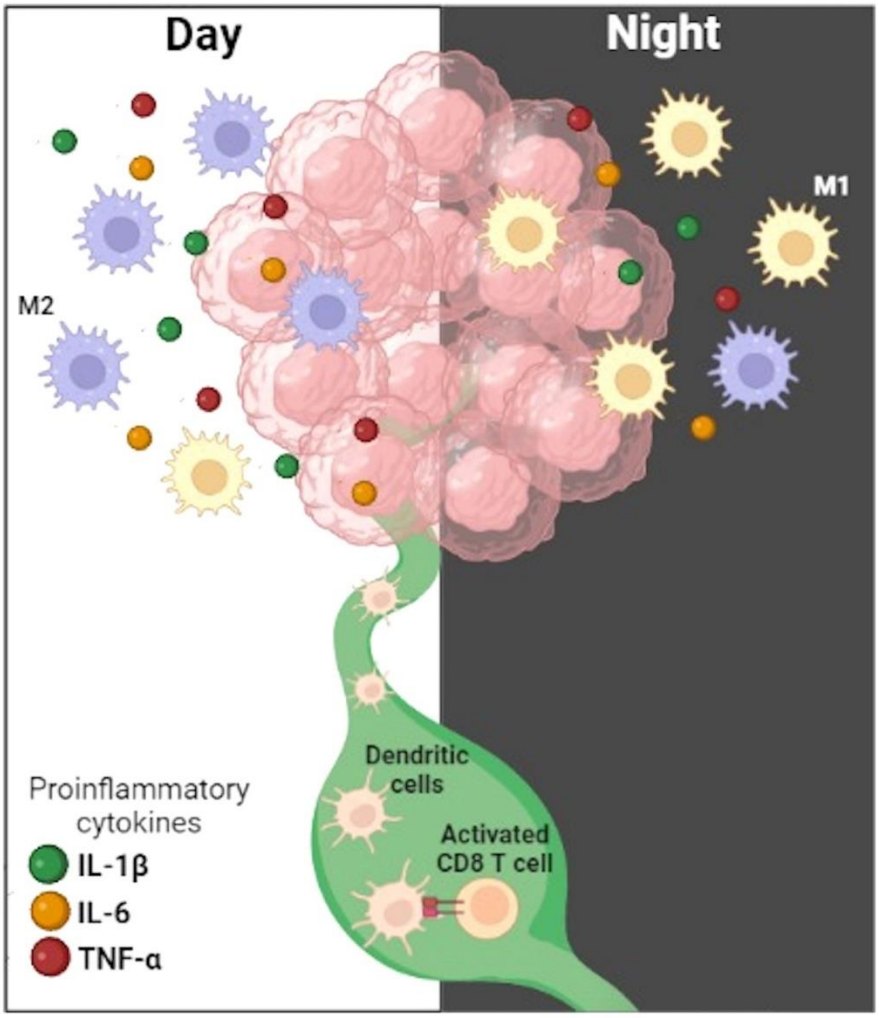

Figure 1. Tumor immune response varies during the LD cycle. Inside the tumor M2 macrophages, along with pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, are increased during the day, while M1 macrophages are higher during the night. Also, Dendritic cells migrate to the tumor-draining lymph node by the end of the day to activate CD8 T cells.

Role of macrophages in the circadian regulation of the tumor microenvironment

The tumor microenvironment

The TME is composed of different types of cells, including fibroblasts and leukocytes, and the extracellular matrix. It comprehends a hypoxic and acidic environment which promotes tumor cell necrosis and release of damage-associated molecular patterns (DAMPs) (19). This DAMPs lead to chronically inflamed areas, in which fibroblasts, tumor cells and immune cells produce pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-17, TNF-α), chemokines (e.g., CCL2, CXCL5, CXCL12) and growth factors (e.g., TGF-β, GM-CSF, VEGF). All of these allow recruiting new leukocytes to the tumor site, differentiating to an anti-inflammatory state, and allowing the tumor to escape from immune surveillance [6]. Among the leukocytes previously mentioned, the most abundant in the TME are the TAMs [19].

It is intriguing that clock genes do not exhibit circadian expression in a murine melanoma model [14, 20]. Interestingly, the synchronization of the circadian clock, by intratumoral injection of low doses of dexamethasone, induces a decrease in the tumor growth rate. However, the immune infiltration of monocytes, DC and lymphocytes did not change after synchronizing [20]. These data show the relevance of the circadian synchronization of the TME and point to the need of a more thorough characterization of specific profiles of immune cells, like TAMs.

Macrophages have an intrinsic clock

Macrophages are capable of responding to environmental cues that allow them to adopt different phenotypes. In mice, it has been reported a higher activity of macrophages (phagocytosis and cytokine secretion) when they are extracted during the day both from peritoneal exudate or spleen [21, 22]. In spleen, both M1 and M2 macrophages are higher during the day while they decrease during the night [14]. Moreover, macrophage response to *in vivo* stimulation is also regulated in a circadian and tissue-dependent manner. There is evidence showing an enhanced pro-inflammatory activation of both peritoneal and splenic macrophages when stimulated during the day-night transition [23, 24], [25]. Circadian regulation of macrophage function is also evidenced by the loss of rhythmic patterns in these cells in macrophage-specific Bmal1$^{-/-}$ mice [24]. Additionally, IL-10 expression is also controlled by Bmal1 [26]. All of these show that macrophages have an intrinsic clock that regulates not only its presence, but also its own activation in a circadian way. Moreover, this data is relevant since the basal circadian differences in macrophages could modulate the early immune response to tumor cells, which could impact the prognosis to cancer.

Macrophages and circadian regulation of the tumor microenvironment

As previously discussed, TAMs can differentiate to a pro-inflammatory state (M1), or get a tissue repair/anti-inflammatory profile (M2), depending on the signals present in the TME. Cytokines like INF-γ induce differentiation to M1 macrophages, while IL-10 or IL-4 governs the differentiation to M2 cells. In addition, pro-inflammatory cytokines, mainly IL-1β and TNF-α, are usually secreted by M1 macrophages, while IL-10 and TGF-β are secreted by M2 [27]. As previously mentioned, in murine melanoma, IL-1β, IL-6 and TNF-α show a circadian expression, with levels that are higher during the day decrease during the night [14]. As also mentioned, macrophage profile changes during the day in a tissue-dependent way. In the tumor, the population of M1 macrophages increased during the night, while M2 macrophages were higher during the day [14]. Interestingly, the M1/M2 ratio showed higher values during the night under LD conditions. Since the M1/M2 ratio has been associated with cancer prognosis [8], the daily pattern in this ratio indicates an anti-tumor immunity favored at night and an enhanced tumor escape during the day [14]. On the other hand, there is an apparent contradiction between the time of higher cytokines levels and the macrophages profiles in the tumor; however, it might be related to the presence of other cytokine-producer cell types or to the time it takes to synthesize and secrete these molecules.

In addition, data suggest a strong effect of chronic circadian desynchronization on the immune system in the TME. In mice maintained under CJL conditions, the mentioned daily patterns observed in LD conditions were disrupted [14]. The levels of M1-infiltrated cells were reversed and dampened, while the levels of M2 macrophages lost their day-night differences and remained constant throughout the whole cycle (Figure 2). Interestingly, the rhythm in M1/M2 ratio, which showed higher values during the night under LD conditions, was blunted in CJL animals, favoring a phenotype in which tumor progression is likely favored at all times. In addition, intra-tumor levels of IL-1 and IL-6 switched from a daily cycle variation under LD conditions to a largely invariant level under CJL, and TNF-α levels appeared to be in antiphase in animals maintained under these conditions. Additionally, the higher levels of leukocytes (total CD45$^+$ and CD4$^+$ T cells) observed in the tumors of animals subjected to CJL could be related to the deregulation of macrophage LD-patterns because of their main role as chemokine producers. This change suggests a functional defect in the immune system inside the tumor in mice under circadian disruption, which could be orchestrated by the robust macrophage clock.

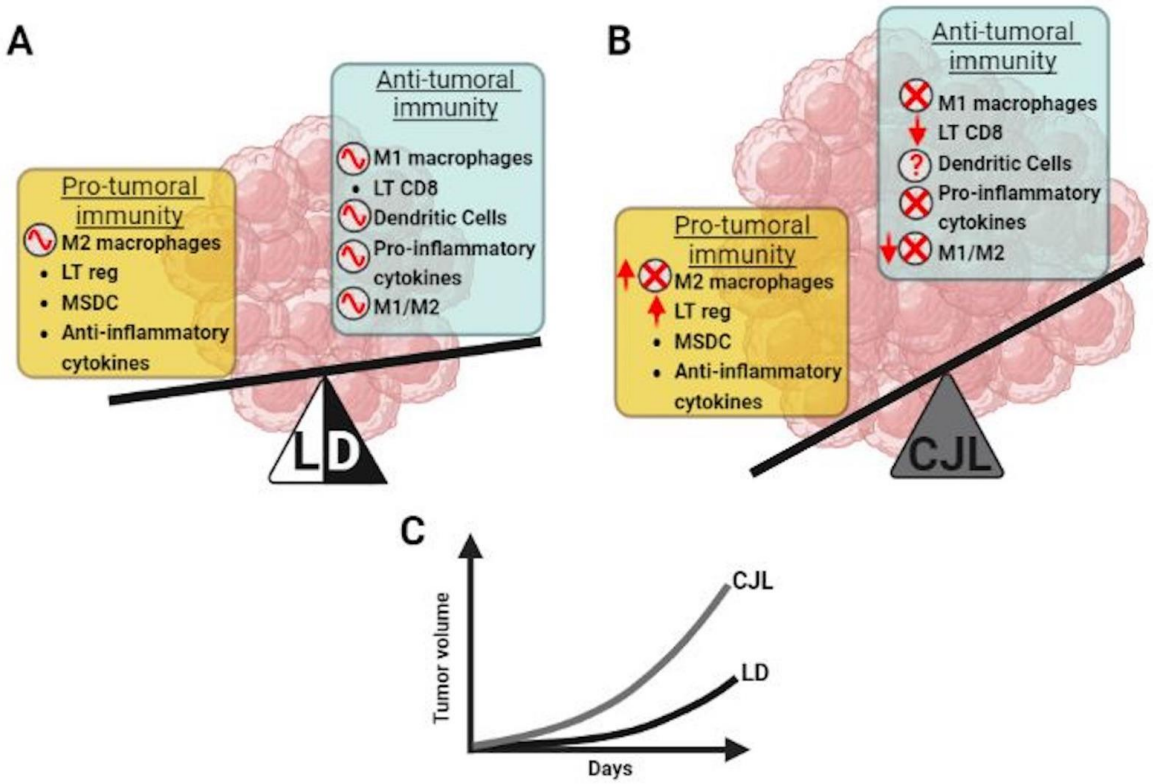

Figure 2. CJL effect in the balance of anti-tumoral immunity in the TME. The anti-tumoral immunity is given by immune populations, such as M1 macrophages, $CD8^+$ T cells and DC, which produce pro-inflammatory cytokines and promotes the elimination of neoplasic cells. On the other hand, the pro-tumoral immunity is composed by M2 macrophages, LT-reg cells and MDSC, among others, accompanied with the secretion of anti-inflammatory cytokines allowing the tumor to escape immune surveillance. In LD conditions (A), many of these cells have a daily pattern, which helps to improve the immune response to tumors. In CJL conditions (B), these daily patterns are lost, accompanied with an increase in the M2 macrophages and LT-reg cells populations and a decrease of the $CD8^+$ T cells and M1/M2 ratio and allowing the tumor to grow faster than in LD conditions (C).

In vitro experiments showed that the M1 and M2 profiles are relatively plastic, since these cells can turn from M1 to M2 or from M2 to M1, depending on the microenvironment and its metabolites [28]. Thus, the daily pattern of these cells could be generated inside the TME. On the other hand, these patterns can be generated by differential recruitment of monocytes/macrophages to the tumor. The recruitment of monocytes to inflammatory sites is mediated by the chemokine CCL2 (also known as MCP-1), an important chemoattractant that is regulated in a diurnal fashion and secreted by macrophages, among other cells [29]. In addition to attracting monocytes, CCL2 is able to induce the differentiation to an M2 profile [30]. Indeed, it was shown that BMAL1 is necessary to generate the rhythmic oscillations in *Ccl2* expression *via* BMAL1/CLOCK binding to E-box motifs in the Ccl2's promoter [29]. In another chemokine pathway, the Cxcr2 inhibitor SB265610 was able to reverse JL-induced metastases and changes in immune cells [15].
One possible mechanism to explain the increased tumoral growth rate in mice under circadian desynchronization could be that the macrophage internal clock is impaired or deregulated, impacting macrophage function, therefore modifying the immune microenvironment inside the tumor.

The tumor presence can also compromise circadian body rhythms
Bidirectional interactions between tumor development and circadian systems have been under intensive study in recent years. Dysfunction in clock-controlled physiologic variables has been observed in cancer patients. The prevalence of sleep-related disturbances (including poor quality of

sleep, insomnia, daytime sleepiness or fatigue) in different types of cancer range from 45% to 80% [31, 32]. In addition, in patients suffering colorectal metastatic cancer, the activity circadian patterns correlate with survival, quality of life, physical and social functioning, fatigue and appetite loss [33]. However, the causes of these rhythm disruptions in cancer are currently unknown. On the other hand, in cancer patients, marked rest-activity circadian rhythms and adequate light intensity improves sleep quality [31] indicating the relevance of chronobiology-driven treatments.

There are very few studies exploring the impact of the tumor presence on the activity circadian pattern in animal models. Mice subject to a murine melanoma model showed a reduction in the amount of the nighttime activity, in the amplitude and in the robustness of the activity rhythm, together with a delay in the activity pattern [34]. Moreover, in an experimental hepatocarcinoma model there was a decrease in the amplitude of locomotor activity and temperature rhythms[35]. Similarly, in a mammary tumor model, there was an increase in the phase angle and in the amount of diurnal locomotor activity, and a decrease in night activity [36], suggesting a direct impact of the tumor presence on the circadian system.

These data are relevant since, as described here, the circadian system modulates the response to the tumor. Thus, if the presence of the tumor disturbs the circadian rhythms, it is expected that it will worsen the patient's prognosis. In other words, the tumor could initiate a vicious circle, in which, at first, it disrupts the circadian system, and next, the altered circadian system worsens the immune response to tumor cells.

Conclusion

The circadian clock regulates daily fluctuations in the immune response, including antitumor immunity. In cancer, tumor cells are surrounded by different cells composed mainly by leukocytes and fibroblasts, generating a tumor microenvironment that facilitates the tumor to escape immune surveillance. When the central clock fails to synchronize with the normal light-dark schedule, the microenvironment gets dysregulated, and the majority of the leukocytes present in the tumor lose their rhythms, enhancing tolerogenic immunity and promoting tumor progression by facilitating tumor escape. In addition, since the presence of the tumor disturbs circadian rhythmicity, it could initiate a vicious cycle where the circadian system gets worse along tumor progression and, as a consequence, the immune response against tumor cells gets compromised and even facilitates the tumor to escape immune surveillance.

In summary, there is compelling evidence of a tridirectional interaction between the immune system, the circadian system and tumor growth, which should be considered in future steps of anti-cancer treatment and recommendations.

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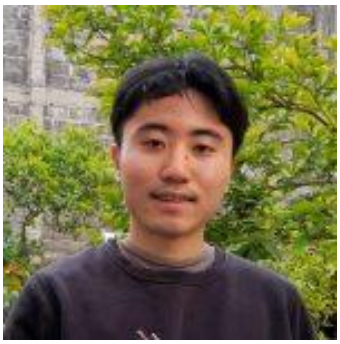

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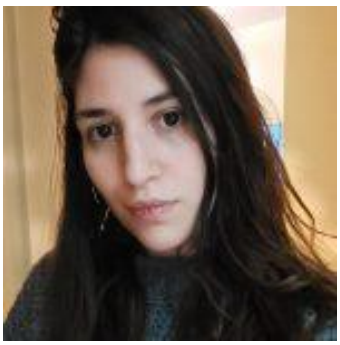

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Diego Andrés Golombek obtained his Bachelor's degree in Biology from the University of Buenos Aires, Argentina. He then went on to complete his Ph.D. in Biology at the same institution. He has made significant research contributions in the area of biological rhythms and circadian rhythms. His work has focused on understanding the molecular and physiological mechanisms behind these rhythms and their impact on various biological processes. He has conducted studies on diverse organisms, including insects, mammals, and humans. Apart from his research, he has been actively involved in science communication and education. writing several popular science books and articles, making complex scientific concepts accessible to the general public. He has received numerous awards and honors for his scientific achievements and his efforts in science communication. Additionally, he is a professor in General physiology at the National University of Quilmes and is currently working as a researcher professor in the Time Interdisciplinary Laboratory (LITERA), San Andrés University.