MACROPHAGES

# Aging alters rhythms in immunity

A new study shows that the transcription factor KLF4 has a role in the maintenance of circadian immune responses. Loss of KLF4 activity contributes to the age-associated immune dysfunction.

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The marvels of modern medicine have improved human health considerably, especially in the developed world, and allowed people to live longer and longer as time goes on. However, the increase in lifespan has presented researchers with new challenges as aging is now a major risk factor in chronic disease[1]. Now, scientists must understand aging further to ensure people can live healthier lives for longer. Immune dysregulation has emerged as a feature of aging that has been linked to chronic disease. Immunosenescence is characterized as the deterioration in immune function with age and contributes to many age-related chronic diseases[2]. In this issue of *Nature Immunology*, Blacher et al.[3] show that circadian rhythms in immunity decline with age and contribute to immune dysregulation in aged mice. The authors identified the transcription factor Krüppel-like factor 4 (KLF4) as the key mediator in circadian immune regulation, the activity of which diminished with age.

Circadian rhythms may be the key to fully understanding the molecular mechanisms of immunosenescence that are associated with aging. Circadian rhythms are endogenous regulatory pathways that repeat every 24 hours and allow organisms to adapt and anticipate daily changes in their environments[4]. The importance of circadian rhythms in immunity have been known since the 1960s, when pioneering work from Halberg et al.[5] demonstrated that survival against endotoxin challenge was dependent on the time of day. As seen with immune responses, the robustness of circadian rhythms also diminishes with age[6]. In the current study, Blacher et al.[3] demonstrate that immune dysregulation in aged mice is due to a loss of circadian regulation of several immune responses despite a functioning core molecular clock[3]. They specifically find that a reduction in KLF4 abundance and rhythmicity in aged macrophages leads to a loss of circadian control and subsequently a diminished immune responses (Fig. 1).

The authors began their study by investigating numerous immune processes that are known to be circadian regulated and how they change with age. As expected, they found strong circadian rhythmicity in monocyte and macrophage trafficking, phagocytosis and survival in a model of endotoxin challenge, all of which were lost in aged mice[3]. Dysregulated immune responses inevitably lead to an increase in infections with aging, an affect that is also seen in older individuals. Building on the work by Halberg et al.[5], the authors found the time-of-day protection provided by circadian rhythms against endotoxin-induced death was completely lost in aged mice, clearly demonstrating the importance of circadian regulation in managing the magnitude of immune responses.

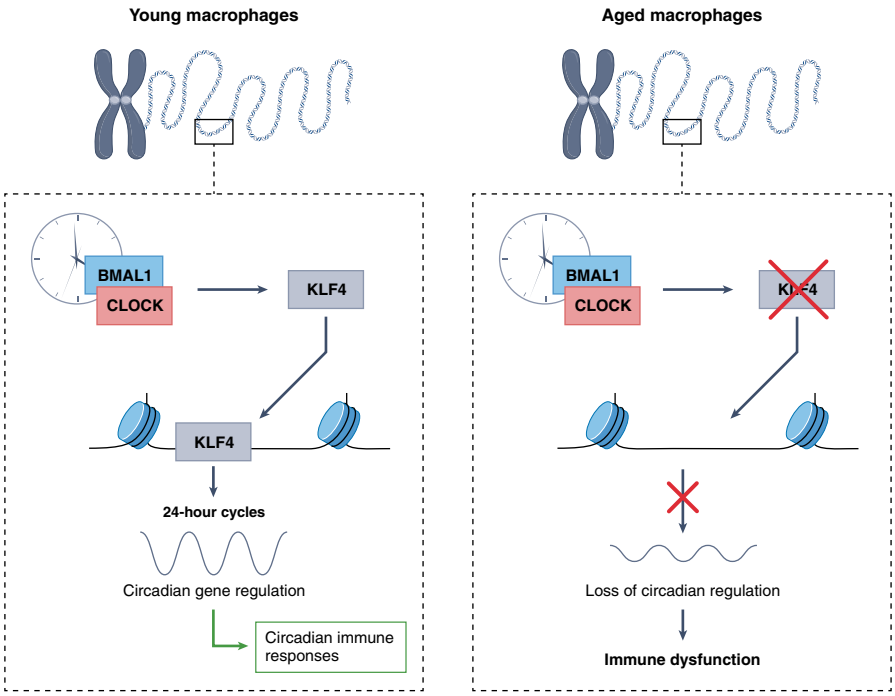


**Fig. 1 | Circadian rhythms have an important role in maintaining macrophage function.** In young macrophages, the core molecular clock maintains healthy immune responses through KLF4. In aged macrophages, KLF4 activity diminishes despite a functioning molecular clock. Loss of KLF4 activity leads to a loss of circadian rhythms of immune responses. The loss of rhythmicity contributes to the immune dysfunction reported in aged macrophages.

To understand the molecular mechanisms behind the loss in circadian rhythmicity, Blacher et al. performed an extensive time course, taking peritoneal macrophages every 4 hours across a 24-hour period and analyzing gene expression using RNA sequencing (RNA-seq)[3]. Using multiple algorithms to identify oscillating gene patterns, they found significantly fewer genes with circadian patterns in aged mice compared to young mice. The authors found that phagocytosis-associated genes within the coordinated lysosomal expression and regulation (CLEAR) network of genes were significantly decreased, explaining the observed loss in circadian rhythmicity of phagocytosis in macrophages. Interestingly, although the overall number of genes with

a circadian pattern was lower in aged mice, those encoding the core molecular clock components, such as brain and muscle arnt-like protein 1 (BMAL1), circadian locomotor output cycles kaput protein (CLOCK) and nuclear receptor subfamily 1 group D member 1 (NR1D1), maintained their circadian rhythms in spite of aging.

The core molecular clock genes are a set of genes that coordinate a complex transcription and translation feedback loop (TTFL). In brief, BMAL1 and CLOCK heterodimerize to drive gene expression, including their own repressors period (*PER1*, *PER2* and *PER3*) and cryptochrome (*CRY1* and *CRY2*) genes. Once the PERs and CRYs reach a critical concentration, they dimerize and translocate from the cytosol to the nucleus, where they inhibit the function of BMAL1 and CLOCK. This whole process takes approximately 24 hours (ref. [4]). As there was no change observed in the TTFL, the authors reasoned that loss in circadian rhythms must occur somewhere downstream of the core molecular clock[3].

The authors hypothesized that a potential molecular mechanism that disrupts circadian rhythms downstream of the TTFL could be a change in chromatin accessibility in aged mice[3]. To investigate this, the authors performed assay for transposase accessible chromatin with high-throughput sequencing (ATAC-seq) on peritoneal macrophages isolated from young and aged mice every 4 hours for 24 hours. The authors found that chromatin accessibility at rhythmic loci were not significantly different between young and aged macrophages. Subsequently, the authors speculated that chromatin accessibility itself could oscillate in a daily pattern, but this proved not to be the case. Analysis using the JTK_CYCLE algorithm on ATAC-seq peaks in young and aged macrophages failed to find any circadian rhythmicity in chromatin accessibility over a 24-hour period.

The presence of circadian rhythms in the core clock machinery in young and aged macrophages, combined with the lack of any difference in chromatin accessibility, suggests that *trans*-acting transcription factor(s) must be present to explain the loss of circadian rhythms in aged macrophages. The authors came up with three criteria to identify *trans*-acting transcription factors within their datasets[3]. The first criterion is that the *trans*-acting transcription factor(s) must differentially bind accessible chromatin between young and aged macrophages. The second is that they must differentially bind genes that are circadian. Finally, the third is that the rhythmic expression of the *trans*-acting transcription factor(s) is lost in aged macrophages. Following investigations, KLF4 was identified as the only transcription factor that meets all three of the criteria for the hypothesized *trans*-acting transcription factor.

The authors then investigated KLF4 further[3]. Short-hairpin RNA (shRNA) targeting BMAL1 in vivo showed that loss of BMAL1 also led to a reduction in KLF4 abundance, placing KLF4 as a direct target of molecular clock. In vivo knockdown of KLF4 with shRNA in peritoneal macrophages in young mice also led to a loss of circadian phagocytosis, highlighting that absence of KLF4 alone is sufficient to cause circadian disruption. This study[3] clearly shows the role of KLF4 in circadian regulation. Interestingly, KLF4 binds to a novel motif to execute these functions — a motif distinct to the motif that KLF4 binds during its more well-known role in cellular re-programming. Data mining from the UK Biobank showed that individuals with mutations in KLF4 were at an increased risk of developing *Escherichia coli* infections. Overall, the work by Blacher et al.[3] provides insights into how circadian disruption can contribute to diminished immunity with age. The authors identified KLF4 as a key transcription factor in the process, and there is no doubt that this paper will lead to further studies on KLF4 for its role in aging, circadian rhythms and immunity.

An interesting finding from the study is that the core molecular clock remains completely functional within aged macrophages, with BMAL1 and other circadian regulators maintaining their 24-hour rhythmicity. Despite a functional core molecular clock, circadian oscillation of KLF4, and subsequent immune processes are lost, suggesting that an intermediate undescribed KLF4 regulatory pathway may exist that is also disrupted by aging. This study focused on identifying genetic regulatory pathways controlled by the molecular clock, but there may potentially be genetic-independent pathways that could contribute to diminished KLF4 activity. One potential pathway that could link circadian rhythms to KLF4 and is known to be dysregulated in aging is mitochondrial dynamics. Mitochondrial dynamics are circadian and KLF4 regulated, known to become dysfunctional with age and are emerging as important regulators of immunity[7–10]. During aging, it has been reported that there is increased mitochondrial fragmentation or reduced mitochondrial metabolism, both of which could be regulated by KLF4 (ref. [11]).

Other potential non-genetic pathways that may regulate KLF4 activity are post-translational modifications, dysregulated antioxidant response or altered metabolism[10,12]. Although Blacher et al.[3] provide novel insights into the links between circadian rhythms, aging and immune function, questions around diminished KLF4 activity in aging remain. Future research will probably focus on identification of the mechanism of loss of circadian rhythms in aged macrophages as their core clock remains functional. If identified, the process that causes diminished circadian rhythms with age could be targeted therapeutically to boost the immune system in older people. Understanding how to boost circadian rhythms to modulate immune responses in the elderly has the potential to overcome immunosenescence, allowing us to live longer and remain healthier as we age. ❐

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## Competing interests

The authors declare no competing interests.