### Title

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### Journal

Trends in Immunology, 43(4)

### ISSN

1471-4906



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### Publication Date

2022-04-01

### DOI

10.1016/j.it.2022.02.005

Peer reviewed

HHS Public Access

Author manuscript

*Trends Immunol*. Author manuscript; available in PMC 2022 November 22.

Published in final edited form as:

*Trends Immunol*. 2022 April ; 43(4): 265–267. doi:10.1016/j.it.2022.02.005.

# Old macrophages lose their (circadian) rhythm

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## Abstract

Blacher and colleagues have discovered that the circadian patterns of macrophage gene expression and immune function that exist in young mice are disrupted in aging mice. KLF4 was identified as a key transcription factor (TF) regulating rhythmic expression of immune genes, which is lost in old macrophages.

The mammalian immune system is regulated by circadian rhythms that cause diurnal patterns of immune cell trafficking and response to pathogens [1–4]. This includes predictable fluctuations in macrophage number and trafficking [2,4], cytokine secretion [5], and phagocytic capacity [3]. In mice, these changes are correlated with differential survival dependent on the time of day that the bacterial endotoxin lipopolysaccharide (LPS) is administered, a phenomenon first described in 1960 by Halberg and colleagues and recently observed in infection models [1–4].

Previous studies focused on young adult mice. However, aging causes increased susceptibility to infection, although the mechanisms underlying this age-related decline in immune function are not well understood. By examining aging mice (18–20-months old), Blacher and colleagues [6] discovered that the diurnal flux of several immune phenotypes disappeared, including fluctuations in spleen macrophage numbers, phagocytic capacity of peritoneal macrophages, and survival in response to LPS challenge, compared with young mice (2–3-months old). These results suggest a link between disruption of the circadian rhythm and age-dependent immune decline.

Analysis of RNA isolated from peritoneal macrophages every 4 h revealed that rhythmic gene transcription was drastically disrupted in older mice, with ~90% fewer genes displaying a diurnal transcription pattern compared with younger mice. Aged macrophages exhibited a significant reduction in the transcription of immune function genes, including a loss of rhythmic expression of phagocytic pathway genes, such as *Mrc1* and *Gba*, compared with young macrophages. Of note, the authors found that core ‘clock’ genes, such as *Clock* and *Arntl1* (encoding BMAL1), which regulate circadian transcription, maintained their periodicity in old macrophages, indicating that mechanisms other than disruption of clock genes caused the observed age-dependent loss of rhythmic gene expression.

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Declaration of interests

None declared by authors.

Blacher *et al.* also assessed chromatin accessibility through ATAC-seq at time points matched to the RNA-seq analysis to understand what other factors might be underlying the disrupted gene expression patterns in aged peritoneal macrophages; while global chromatin accessibility was lost in aged macrophages, these changes did not occur at peaks (i.e., sites of accessible or 'open' chromatin) at, or closest to, the transcriptional start sites of rhythmic genes. Moreover, there was no rhythmic pattern associated with chromatin accessibility peaks in young macrophages, indicating that oscillations in nucleosome positioning within chromatin were unlikely to represent a driver for rhythmic transcription; this led the authors to hypothesize that differential TF binding might regulate rhythmic gene expression, and loss thereof, in aged macrophages.

Furthermore, using a computational approach to predict TF binding by identifying TF motifs within differentially accessible regions of chromatin, the authors unveiled KLF4 as a potential driver of rhythmic gene expression in macrophages; indeed, regions differentially accessible between young and aged macrophages were enriched for KLF4 motifs. In addition, KLF4 and its target genes exhibited rhythmic gene expression in young macrophages, which was abolished in aged ones (Figure 1). Moreover, knock down of *Arntl* resulted in loss of *Klf4* rhythmicity in young macrophages, positioning KLF4 downstream of the CLOCK:BMAL1 complex. However, since the core clock machinery remained intact in aged macrophages, the authors posed the key question of what other factors upstream of KLF4 might drive the loss of the *Klf4* oscillatory gene expression pattern with age.

The authors functionally validated the importance of KLF4 in modulating rhythmic immune phenotypes using *ex vivo* and *in vivo* short hairpin (sh)RNA knockdown experiments in differentially aged mouse macrophages. *Klf4* knockdown decreased phagocytosis in both young and aged macrophages *ex vivo*. Additionally, after *Klf4* knockdown *in vivo*, young peritoneal macrophages lost the rhythmic expression of phagocytosis genes (*Gba* and *Rab3d*) and diurnal pattern of phagocytosis. This suggested that loss of *Klf4* caused young macrophages to adopt rhythmic and gene expression characteristics of old macrophages, supporting the idea that KLF4 is a key driver of rhythmic phagocytosis in young macrophages that is disrupted with aging. Given that KLF4 can affect many aspects of macrophage function [7–9], experiments directed at investigating the contribution of *Klf4* rhythmicity to its TF activity are of interest. For example, it remains unknown whether KLF4 exhibits rhythmic protein expression and whether KLF4 drives rhythmic expression of its target genes via oscillatory binding, which could be addressed by assessing KLF4 binding using ChIP-seq. Alternatively, KLF4 may be required to maintain accessibility through its pioneer TF activity [10] for other factors, such as the CLOCK:BMAL1 complex, thus driving oscillatory expression of immune response genes. Therefore, a reduction in KLF4 activity in aged macrophages might lead to loss of chromatin accessibility (as observed by the authors) [6], as well as loss of rhythmic expression of immune response genes in these cells.

Finally, Blacher and coworkers addressed the clinical relevance of these findings using biobank data to assess the impact of genetic variation at the *KLF4* human locus. A remarkable finding was that carriers of a particular genetic variant (rs2236599) that results in an adenine-to-guanidine substitution in KLF4 were more at risk of *Escherichia coli*

infection, and more likely to succumb to infection-related mortality than were noncarriers [6]. Furthermore, while aged individuals were more likely to develop an *E. coli* infection than were young individuals in the general population, this difference was less pronounced between young and old carriers of the variant, likely because they were already more immunologically susceptible.

Therefore, these correlative studies show a link between the *KLF4* rs2236599 variant, infection risk, and age, warranting further research into how this variant might impact KLF4 activity and macrophage function.

Overall, these studies by Katrin Andreasson's group contribute to our understanding of how the immune system can be disrupted during aging and shed light on the molecular underpinnings of circadian control in macrophages. Efforts to understand the contribution of KLF4 and circadian gene regulation in inflammatory diseases and the age-associated decline of immune function in infection and cancer might aid the future clinical application of these findings.

## Acknowledgments

D.C.H. is supported by the National Institutes of Health (GM128943-01, CA184043-03, and AI151123), the Pew-Stewart Scholars for Cancer Research, and the American Cancer Society Research Scholar Award. H.M.M. is supported by a CRI/Merck Postdoctoral Fellowship (CRI Award #4045), an American Australian Association Graduate Education Fund Scholarship, and a Salk Women & Science Special Award.

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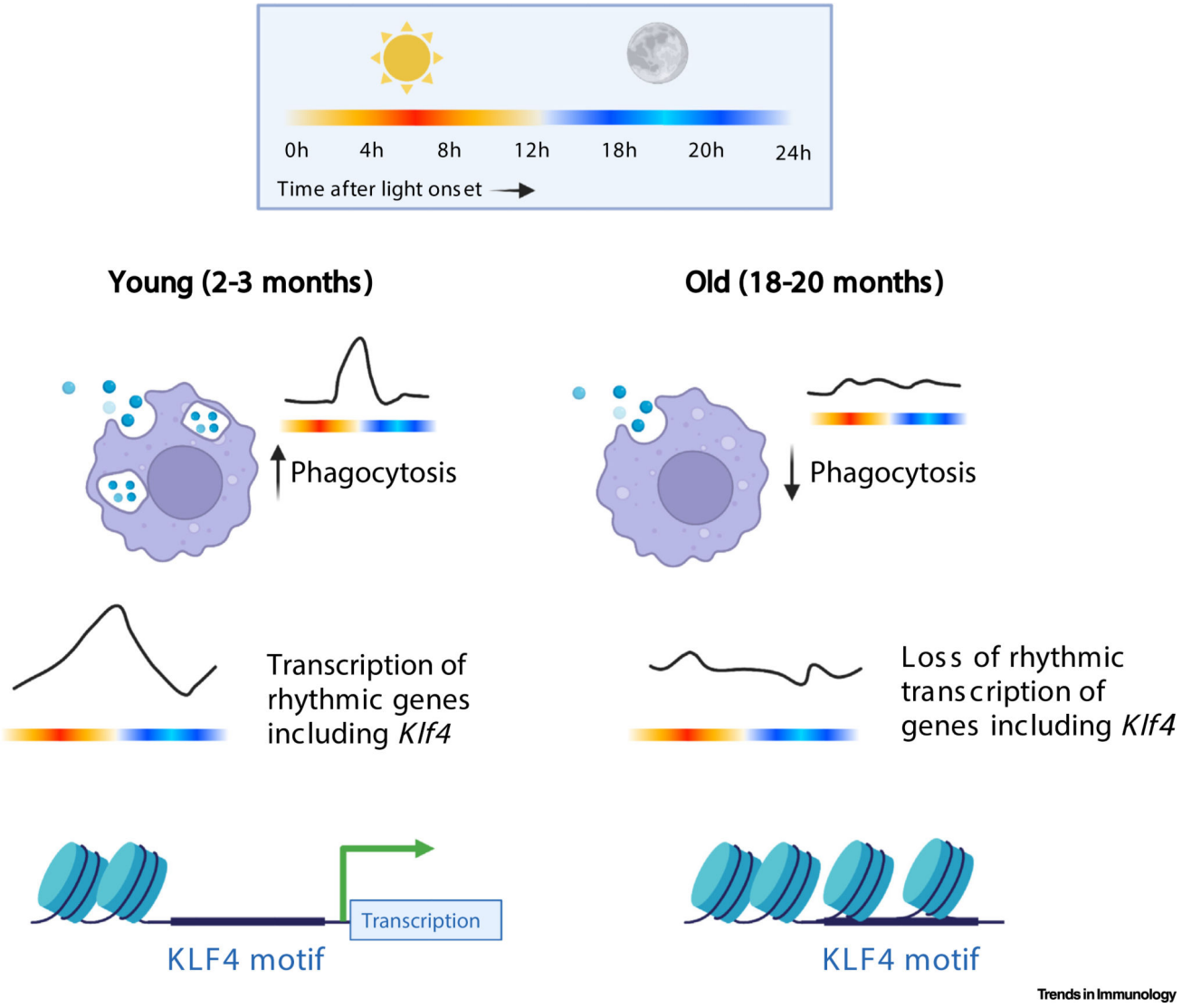


**Figure 1. Aged macrophages lose rhythmic function, gene expression, and accessible chromatin at KLF4 transcription factor (TF) motifs in mice.**

Peritoneal macrophages were isolated from young (2–3-months old) and old (18–20-months old) mice every 4 h during a 12 h/12 h light/dark cycle [6]. Corroborating previous studies, young macrophages displayed a diurnal pattern of phagocytosis; however, older macrophages lost this rhythmic pattern of immune function and displayed reduced phagocytic function compared with young macrophages. Several hundred genes, including *Klf4*, displayed rhythmic transcription in young macrophages but were not rhythmic in old macrophages. KLF4 motifs were identified as enriched among accessible chromatin in young macrophages compared with old ones and were associated with genes that showed distinct oscillatory gene expression patterns between age groups [6]. Figure created with BioRender (Biorender.com).