Commentary | Received 26 January 2026; Revised 18 March 2026; Accepted 20 March 2026; Published 25 March 2026
https://doi.org/10.55092/exrna20260002

A CLOCK mRNA-targeting long non-coding RNA guides innate immunity training in tuberculosis

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Highlights:

- Circadian rhythm influences innate immunity.
- Innate immunity training significantly improves the effectiveness of vaccinations.
- CLOCK mRNA stabilization by lncRNA enhances transcription of innate immune genes.

Abstract: Recent years have brought the groundbreaking discovery of trained innate immunity, characterized by enhanced monocyte, macrophage and NK cell microbicidal activity. A functional shift of the involved cells results from metabolic rewiring and epigenetic modifications that, among others, are driven by long non-coding ribonucleic acid (lncRNA)-induced effects. However, many questions remain unanswered regarding the precise molecular pathways that ensure trained immunity. In this Commentary article, we aimed to present a comprehensive summary of the key findings from a recently published study that identified a role for lncRNA in monocyte training in the context of tuberculosis. Interestingly, this extracellular vesicle-transferred lncRNA has been shown to stabilize clock circadian regulator (CLOCK) mRNA and, in turn, augment its translation. Consequently, CLOCK-induced histone acetylation upregulates the expression of immune and circadian genes in trained monocyte-derived macrophages. These lncRNA-induced effects were demonstrated to increase antimicrobial resistance after immunization with the Bacillus Calmette-Guérin (BCG) vaccine as well as during unrelated infections. These latter features are desirable characteristics of trained immunity.

Keywords: circadian rhythm; epigenetic regulation; extracellular vesicles; lncRNAs; monocytes; macrophages; trained innate immunity; tuberculosis

1. Introduction

Long-lasting immunological memory is a remarkable feature of adaptive immune cells, and more specifically B2 and Tαβ lymphocytes, enabling them to respond extremely quickly and precisely to secondary contact with a given antigen [1]. However, recent years have brought the groundbreaking discovery, which revealed that certain activating stimuli are able to train innate immune cells, such as

monocytes, macrophages and NK cells, to be more effective in fighting encountered pathogens. This phenomenon has been termed trained immunity and defined as a long-lasting, stable functional reprogramming of the innate immune cells, induced by an exogenous or endogenous stimulus, which results in a heightened effector response to an unrelated secondary stimulation of the cell that in the meantime has returned to a resting state [2].

It is worth noting that innate immune cell training is typically initiated by pathogen and damage-associated molecular patterns (PAMPs and DAMPs, respectively) that stimulate pattern recognition receptors (PRRs). In addition to the mechanisms that drive heightened antimicrobial innate response, trained immunity also develops during the course of chronic inflammatory diseases, characterized by the release of DAMPs [2,3].

Trained innate immune cells exhibit fixed functional shift resulting from metabolic rewiring and epigenetic modifications [4]. Epigenetic regulation of gene transcription includes changes in DNA methylation pattern, histone modifications (mostly by methylation and acetylation), remodeling of the three-dimensional chromatin structure as well as transcription of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) [5,6]. The latter encompass RNA molecules of more than 500 nucleotides that mostly are transcribed by RNA polymerases I-III and synthetized on an intron template [7]. Interestingly, lncRNAs are currently considered to link metabolic and epigenetic reprogramming in trained innate immune cells to facilitate the transcriptional priming of the inflammatory genes [8]. However, our knowledge on the involvement of lncRNAs in trained immunity is still in its early stages [9].

On the other hand, it should also be stressed that lncRNAs may be considered promising epigenetic regulators of overactivated macrophages under inflammatory conditions, as suggested by some recent studies [10].

2. Vaccination-induced innate immune cell training from a circadian rhythm perspective

As mentioned, certain PAMPs are considered to be the most effective in promoting trained immunity. Therefore, it has been speculated that vaccines, in addition to inducing memory cells of adaptive immunity, may also train innate immune cells. Indeed, in humans, trained immunity was first demonstrated to provide heterologous protection against infections and was elicited by some vaccines, especially those containing live, attenuated bacteria. So far, the best-studied vaccine in this area is the Bacillus Calmette-Guérin (BCG) vaccine. Numerous studies have shown that BCG vaccination is efficient not only against tuberculosis but also against other infections due to the ability of live, attenuated *Mycobacterium bovis* to enhance macrophage antimicrobial response [3,4]. This opens up a completely new perspective on the development of modern vaccines, the main goal of which will be to induce a robust trained immunity [11]. To accelerate the development of such vaccines, however, we need to thoroughly understand the mechanisms underlying the training of innate immune cells.

Moreover, the circadian rhythm, intracellularly controlled by clock circadian regulator (CLOCK) gene, is currently considered an important regulator of immunity [12], starting from the maternal-fetal interaction [13], to a major impact on the vaccination efficiency in both adaptive and innate compartments [14–16]. Besides, recent studies point to circadian variability of the immune system as a potential adaptive strategy for resistance to infections [17]. Thus, when optimizing vaccination strategies, the circadian rhythm should also be taken into account [18]. Accordingly, some studies suggest that BCG-induced trained immunity is associated with polymorphisms in the *RORA* gene

encoding the transcription factor RORα, which, among others, is involved in the regulation of the circadian rhythm [19]. However, there is still a significant gap in our knowledge in this area, and research is primarily limited to tuberculosis, while the suggested mechanisms appear to be universal to mammals. Such speculation is supported by the fact that some other live attenuated vaccines, like measles-mumps-rubella (MMR) vaccine, also induce cross-protection against various related infectious agents. Thanks to research such as the one commented in this article, it is now widely known that this is primarily the result of innate immune cell training, which completely changes our view of the composition and expected effectiveness of modern vaccines [20]. Yet, we still have a long way to go towards fully understanding all these complex relationships and the underlying molecular pathways.

Finally, miRNAs and lncRNAs are often transmitted by extracellular vesicles (EVs), including exosomes [21]. The question then arises whether these transferred epigenetic regulators are able to train target cells in a manner analogous to that of those produced intracellularly [22].

Accordingly, the recently published article by Yu *et al.* [23] provides a thorough answer to some of these issues by describing the mechanism of CLOCK-targeted, lncRNA-mediated epigenetic regulation of monocytes and macrophages in the context of tuberculosis infection. Importantly, the authors' findings appear to explain the clinical observations that morning BCG administration induces trained immunity more potently than evening vaccination in correlation with increased CLOCK expression [14], thereby supporting the chronovaccination concept [18].

3. A lncRNA-driven CLOCK rhythm epigenetically shapes monocyte and macrophage response in tuberculosis

Mounting clinical evidence has suggested that tuberculosis resisters (*i.e.* individuals not developing an active or latent infection after boosted exposure to *Mycobacterium tuberculosis*) show no signs of acquiring antigen-specific adaptive immunity. This draws scientists' attention to the involvement of trained innate immune cells capable of early removal of the invading pathogen. Along these lines, resisters' circulating monocytes express shifted transcriptional status due to epigenetic histone acetylation, which augments their antimicrobial activity. These observations prompted Yu *et al.* [23] to search for the bloodborne mediators inducing monocyte training against *Mycobacterium tuberculosis*.

First, blood sera collected from healthy individuals, actively or latently infected patients, and tuberculosis resisters were tested *in vitro* for their modulatory potential on monocytes from healthy controls. This approach unraveled that only resisters' sera upregulate antimicrobial activity of classical monocytes, and this effect was abolished by EV depletion. Therefore, in the next step, the authors had employed transcriptional profiling to characterize the changes induced by resisters' serum EVs in monocytes, and observed that differentially expressed genes were enriched for biological processes related to innate immune cell activation, cellular circadian rhythm, and cellular metabolism, altogether confirming that monocytes underwent immune training. It also caused monocytes to differentiate into macrophages exerting augmented microbicidal activity against various pathogens.

Depletion of individual EV components followed by profiling of extracellular RNA repertoires allowed the identification of lncRNA as a regulator of monocyte activity, while prediction of molecular interaction pairs and siRNA-mediated functional validation revealed RNA-binding protein fragile X mental retardation syndrome-related protein 2 (FXR2) as a dominant target. FXR2 involvement in circadian regulation and demonstrated upregulation of genes related to circadian rhythm prompted

researchers to hypothesize that lncRNA may induce trained immunity by modulating monocyte circadian clock machinery. Among FXR2-targeting lncRNAs found in resisters' serum EVs, *AL118516.1* (also known as *CTA-29F11.1*) was shown to most significantly increase CLOCK expression in monocytes. Therefore, it was named tuberculosis resister-derived CLOCK regulator 1 (TRCR1). Importantly, a number of *in vitro* experiments have shown that TRCR1 upregulates CLOCK translation after forming a stabilizing complex with its mRNA and FXR2. In turn, this increases CLOCK's intrinsic histone acetyltransferase activity in monocytes, resulting in augmented expression of immune and circadian related genes in monocyte-derived macrophages triggered by histone acetylation at H3 lysine 9 and 14 (H3K9ac/H3K14ac).

Mechanistically, TRCR1 was found to bind to C-terminal domain of FXR2, which recruits CLOCK mRNA to the same binding site and significantly prolongs its half-life. In turn, CLOCK overexpression greatly increases H3K9ac/H3K14ac enrichment at promoters of key cytokine and autophagy-related genes (TNF, IL1B, IL6 as well as ATG2A, ULK1, TBK1, respectively), which induces the long-lasting trained phenotype in macrophages.

In addition, database search revealed high expression of TRCR1 in airway epithelial cells, and *in vitro* experimental validation proved that an exposure of the latter cells to *Mycobacterium tuberculosis* increases the expression of this lncRNA, which is then released in EVs. After engulfment by monocytes, EV-transmitted TRCR1 induces CLOCK-dependent epigenetic remodeling, which, as a consequence, greatly augments the antimicrobial immunity mediated by monocyte-derived macrophages, as confirmed in *in vivo* mouse model of *Mycobacterium tuberculosis* infection. These experiments also demonstrated that TRCR1 enhances the efficacy of BCG vaccination.

The studies by Yu *et al.* [23] have significantly contributed to our current understanding of the mechanistic basis of trained immunity and have demonstrated that EV-transferred lncRNAs are functionally active in targeted cells (Figure 1). Furthermore, these results indicate the possibility of systemic induction of a trained phenotype in innate immune cells in order to provide enhanced microbicidal activity through intravenous administration of epigenetic regulators.

4. Conclusion

Although limited solely to tuberculosis, this research unravels a novel pathway of epigenetic regulation mediated by EV-transported lncRNA (Figure 1). This is of critical importance while considering the modern vaccination strategies aiming at training innate immune cells, allowing us to deepen our current understanding of complex cellular interactions and epigenetic regulation. Finally, these findings support the process of optimizing vaccination strategies for circadian rhythm. This is a very promising approach to improve vaccination effectiveness in the global population.

As summarized elsewhere [24,25], the endogenous biological clock regulates the complex rhythmicity of the immune system, optimizing its functioning to maintain immune homeostasis and disease resistance. Therefore, insights from studies on the interregulation of circadian rhythm and immune cell activity will also be applied to efforts to restore normal immune function in non-infectious diseases, including cancer, inflammatory and autoimmune diseases [26,27], and to greatly enhance the efficacy of immunotherapies [28,29]. In this regard, the multifaceted epigenetic functions of immunoregulatory lncRNAs make them promising candidates for such approaches [30], especially in the context of macrophage training and circadian rhythm [31]. Thus, according to the commented article

and other observations [32], macrophage training perpetuated by lncRNA-driven epigenetic regulation of circadian rhythm is of great importance in general immunoregulation, not only in vaccinology.

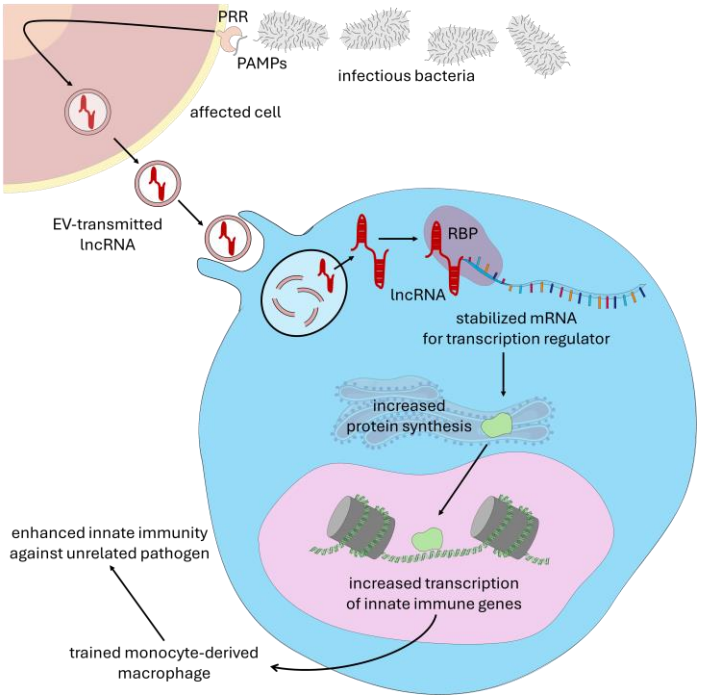

Figure 1. A putative pathway of epigenetic regulation of innate immune cells by lncRNAs transported by extracellular vesicles, postulated on the findings of Yu *et al.* [23]. Some of the icons were adopted from smart.servier.com, and were used in compliance with the terms of the Creative Commons Attribution 3.0 Unported License. EV, extracellular vesicle; lncRNA, long non-coding ribonucleic acid; mRNA, messenger RNA; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptor; RBP, RNA-binding protein.

However, before epigenetics-based approaches can be implemented into routine clinical practice, certain challenges need to be clarified. Among them, perhaps the most difficult challenge is limiting the activity of epigenetic regulators to selected cells after systemic administration to increase clinical efficacy and reduce potential side effects. This can be achieved, for example, by selectively targeting extracellular vesicles that transport them to appropriate recipient cells using a variety of mechanisms and modifications [33]. The next challenge is to determine and standardize the effective dose and the safest route of administration of epigenetic regulators. It is also equally important to understand the precise effects of epigenetic interactions, as the induced regulatory effect may lead to the activation of other molecular pathways in the cell, which may contribute to the development of resistance to epigenetic drugs or even produce the opposite effect than expected [34].

Altogether, the commented research, as well as any basic study in this field, constitutes an element of the foundation bringing us closer to the widespread use of epigenetic modulators in both immunoregulation and strengthening the body's immunity.

Declaration of generative AI and AI-assisted technologies

The authors did not use generative AI or AI-assisted technologies in the writing of this manuscript.

Acknowledgments

We would like to thank Spencer D. Strobel, MD, for his help in linguistic proofreading of the manuscript. Graphical abstract was created by Krzysztof Bryniarski in BioRender under the license dedicated for the Faculty of Medicine, Jagiellonian University Medical College.

Authors' contribution

Conceptualization, KN and KB; formal analysis, KN, YD and KB; writing—original draft, KN and YD; writing—review and editing, KB. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Katarzyna Nazimek holds the position of Editorial Board Member for *ExRNA* and has not peer reviewed or made any editorial decisions for this paper.

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