Role of N4-acetylcytidine for continuously activating NLRP3 inflammosome by HMGB1 pathway in microglia

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N4-acetylcytidine (N4A) is an organic compound and a metabolite of transferrable ribonucleic acid. Its molecular formula is $C_{11}H_{15}N_3O_6$. Preliminary studies suggest that N4A was mainly found on tRNA and 18S rRNA, while recent studies have shown that there is also a large amount of N4A on mRNA, whose abundance is not even lower than the m7G cap modification carried by mRNA (Arango et al., 2018). The generation of N4A is catalyzed by N-acetyltransferase 10 (NAT10) or its homologous enzyme. N4A is produced by acetylation in eukaryotic RNA and is the only human enzyme with both acetyltransferase and RNA binding activity (Arango et al., 2018). The full transcriptome mapping of N4A shows abundant discrete acetylation regions in the coding sequence. The ablation of NAT10 reduces the detection of N4A at mRNA localization sites and is globally correlated with the down-regulation of tmRNA. N4A is widely distributed in the human transcriptome, and most sites occur in the coding sequence. Compared with unmodified cytosine, N4A increases the thermal stability of Watson-Crick base pair guanosine, thus affecting the interaction with homologous tRNAs during translation. After the release of N4A from tRNA metabolism, it participates in the systematic immune response (Ito et al., 2014).

N4A was once considered as a biomarker for diabetes and colorectal cancer patients, who showed obvious signs of oxidative stress. In a variety of oxidative stress processes, tRNA may be degraded, and N4A can be released from tRNA metabolism, which normally cannot be reused and further degraded, but excreted in urine (Youm et al., 2015). Furman et al. (2017) found that the level of endogenous nucleoside metabolite N4A from tRNA degradation was significantly increased in the elderly with high expression level of inflamosome gene. The ability to regulate and transfer energy gradually decreases as aging, the body metabolism slows down, oxidative stress increases, and metabolites gradually accumulate in plasma. However, the role of these metabolites in human body is not fully understood.

Chronic mild inflammation is related to many aging diseases. Inflammasome can drive chronic inflammation and trigger the maturation of interleukin (IL)-1β under the condition of infectious diseases or oxidative stress (Bai et al., 2018; Zhang et al., 2019). N4A can activate the microglia of the central nervous system and induce the activation of NLRC4 inflammasome. After monocytes treated with adenosine, the expression of *nlrc4* gene was increased and mature IL-1β was released. Because N4A induces cytokine secretion in the presence of ATP, and N4A increases the expression of *NLRC4* gene and protein, it seems that N4A can increase the expression of NLRC4 in monocytes, and then adenine can trigger the activation of NLRC4 inflammasome and the maturation of cytokines (Furman et al., 2017).

There is a high level of oxidative stress in the central nervous system of the patients with neurodegenerative diseases such as Alzheimer's disease (AD) (Bai et al., 2018), and N4A may be released from tRNA metabolism. In addition, it has been shown that N-acetyltransferase 10 can prolong the life of aging mouse model, while N-acetyltransferase 10 can inhibit the production of N4A (Cekic et al., 2016). The level of N4A, an endogenous nucleoside metabolite from tRNA degradation, increased significantly in the elderly with high expression activity of inflammatory bodies. N4A induced and activated nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome to induce IL-1β (Balmus et al., 2018). N4A can activate NLRP3 inflammasome by inducing the expression of NLRP3. It can also promote the assembly and subsequent activation of NLRP3 inflammasome by inducing the expression of *HMGB1* gene. It found that N4-acetylcytidine can activate the immune function of microglia and maintain the activation of NLRP3 inflammasome by inducing the expression of HMGB1 protein; the released HMGB1 can cause N4A to activate nuclear factor kappa B (NF-κB) and induce the expression of *NLRP3* gene; the silencing of *HMGB1* gene can eliminate the activation of *NF-κB* gene stimulated by N4A and inhibit the gene expression of NLRP3 and HMGB1. There is a high level of HMGB1 in the blood of AD patients, suggesting that HMGB1 may be involved in the process of immune neuroinflammation caused by the activation of NLRP3 (**Figure 1**). Our results show that N4A mediated HMGB1 release is necessary for sustained HMGB1 expression through NF-κB signaling and up-regulation of NLRP3 expression. Firstly, inhibition of HMGB1 expression by RNAi eliminated N4A mediated up-regulation of HMGB1, NF-κB subunit and NLRP3 expression levels and HMGB1 release. Secondly, the supernatant of BV2 microglia transfected with specific siRNA against HMGB1 eliminated the up-regulated NF-κB signal transduction and NLRP3 expression of N4A (Duan et al., 2019).

High mobility group (HMG) protein is named for its high migration ability in polyacrylamide gel electrophoresis. HMG can be further divided into three families: HMGA, HMGB and HMGN. HMGB family has HMGB1, HMGB2 and HMGB3 and HMGB1 is the most abundant HMG protein. *HMGB1* gene includes 5 exons and 4 introns (Fonken et al., 2016). Human *HMGB1* gene is located on chromosome 13q12. *HMGB1* gene has a powerful TATA box promoter and contains a silence element. Under general conditions, the expression of HMGB1 is maintained at the basic level. It has been that the pro-inflammatory effect of HMGB1 is mainly through the receptor for advanced glycation end products. NF-κB, mitogen activated protein kinase, protein kinase B, plasminogen activated inhibitor and Cdc42 protein are activated by receptor for advanced glycation end products (Arosio et al., 2016).

The following questions need to be answered: can N4A induce the sustained expression and release of HMGB1 by activating the immune function of microglia? Does N4A mediated NF-κB signaling and NLRP3 expression require HMGB1 protein expression? Therefore, we hypothesized that N4A can maintain the activation of NLRP3 inflammasome by inducing the expression and release of HMGB1. Does N4A mediated HMGB1 expression require activation of NLRP3 inflammasome? The results showed

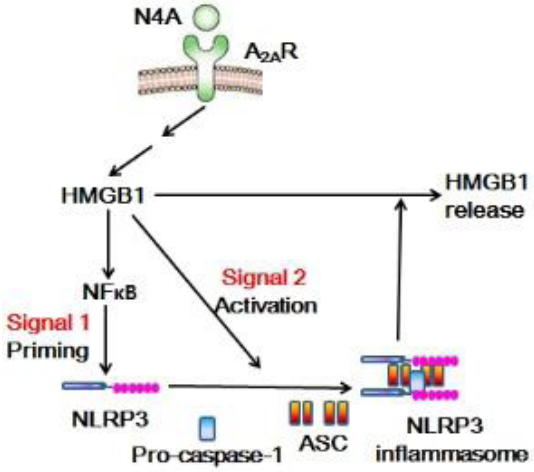

Figure 1 | **Molecular mechanism of N4-acetylcytidine continuously activating NLRP3 inflammasome by HMGB1 pathway in microglia.**
HMGB1 may play an important role in the pathogenesis of AD. N4A may first initiate the NLRP3 inflammasome, and induce the expression of NLRP3 and HMGB1 through signal 1, and then N4A-induced HMGB1 as signal 2 to promote the assembly and subsequent activation of NLRP3 inflammasome. N4A-mediated HMGB1 release was dependent on NLRP3 inflflammasome. A2AR: Adenosine A2A receptor; AD: Alzheimer's disease; ASC: apoptosis-associated speck-like protein containing a caspase-recruitment domain; HMGB1: high mobility group protein B1; NLRP3: nucleotide-binding oligomerization domain-like receptor protein 3.

that N4A promoted the production and activation of NLRP3 inflammasome through HMGB1 in microglia, and silencing the expression of NLRP3 eliminated the up-regulation of HMGB1 expression and release mediated by N4A, indicating that the release of HMGB1 mediated by NLRP3 inflammasome is necessary for maintaining the expression of HMGB1 in N4A (Fonken et al., 2016). Reducing the activation of microglia induced by β-amyloid (Aβ) is considered to be an effective treatment for AD. Falcao et al. (2017) used mouse microglial cell line and solution containing Aβ aggregate mixture to study whether dipeptide vinyl sulfone can attenuate Aβ mediated inflammatory response. The results showed that dipeptide vinyl sulfone could inhibit the expression of HMGB1, NLRP3 and IL-1β induced by Aβ.

HMGB1 may play an important role in the pathogenesis of various neurodegenerative diseases, especially AD. Therefore, it is critical to identify the inducer of HMGB1 expression. Much attentions should be payed on N4A and its similar chemicals in the future. Investigation on HMGB1 signal transduction and endogenous nucleotide metabolites activating inflammasomes in microglia will pave the way for the prevention and treatment of AD and other diseases.

This work was supported by the grant from Guizhou Science and Technology Department Support Plan Project in China, No. Qiankehe-zhicheng [2020]4Y129 (to HB).

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Date of submission: June 9, 2020
Date of decision: August 14, 2020
Date of acceptance: October 11, 2020
Date of web publication: December 12, 2020

https://doi.org/10.4103/1673-5374.301006
How to cite this article: *Bai H, Zhang Q (2021) Role of N4-acetylcytidine for continuously activating NLRP3 inflammosome by HMGB1 pathway in microglia. Neural Regen Res 16(7):1427-1428.*

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C-Editors: Zhao M, Li JY; T-Editor: Jia Y