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N^4-acetylcytidine is required for sustained NLRP3 inflammasome activation via HMGB1 pathway in microglia

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ABSTRACT

Persistent inflammasome activation contributes to chronic, low grade inflammation. However, it is unclear how the inflammasome activation is sustained after initiation. Here we reported that N^4-acetylcytidine (N4A), a nucleoside metabolite, activated microglia and sustained NLRP3 inflammasome activation by inducing HMGB1 signaling. Released HMGB1 through N4A activated NFκB and induced NLRP3 expression. HMGB1 silencing abolished N4A-stimulated NFκB activation, NLRP3 and persistent HMGB1 expression. In addition, inhibiting NLRP3 expression by RNAi abrogated N4A-mediated HMGB1 expression. Lack of NLRP3 inflammasome adaptor named apoptosis-associated speck-like protein containing a CARD (ASC) abrogated N4A-induced HMGB1 expression, NFκB activation, and NLRP3 expression. Taken together, our results reveal a novel role of N4A in activation of NLRP3 inflamasome via HMGB1 feedback.

1. Introduction

Chronic low-grade inflammasome activation plays an important role in neuroinflammation which is associated with aging [1,2] and Alzheimer's disease (AD) and Parkinson's disease (PD) [3,4]. Inflammasome is a complex of subunits including NOD-, LRR- and pyrin domain-containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1. Inflammasome activation requires two signals: signal 1 induces NLRP3 expression, and signal 2 triggers to assembly inflammasome subunits and activate inflammasome. Chronic inflammasome activation requires sustained signals to maintain. However, the mechanisms responsible to produce sustained inflammasome activation remain unclear.

Recent studies have shown that nucleotide signaling mediates immunity by activating inflammasome. During inflammatory conditions, ATP, ADP, UTP or UDP mediate inflammation via signaling at P2-purinergic receptors, or A_{2A} receptor ($A_{2A}R$), or $A_{2B}R$, that elicit proinflammatory immune responses [5–7]. In macrophages, ATP signals through P2X7 purinergic receptors (P2X7Rs) to activate the NLRP3 inflammasome and caspase 1 to trigger the maturation of IL-1β and other cytokines [7]. ATP-induced acute production of IL-1β is significantly resolved within 24 h [8], whereas persistent inflammasome activation is established in a number of chronic inflammatory diseases, suggesting that additional regulatory factors are needed to maintain sustained inflammasome activation. For example, adenosine may maintain persistent inflammasome activation via the $A_{2A}R$ and the HIF-1α pathway in macrophages [9]. However, the mechanisms underlying persistent inflammasome activation in the central nervous system (CNS) are still unclear.

A recent study reported that the level of N^4-acetylcytidine (N4A), an endogenous nucleoside metabolite from the degradation of tRNA, was significantly elevated in older people with high expression levels of inflammasome gene modules. N4A primed and activated NLRC4 inflammasome and NLRP3 inflammasome to induce IL-1β [10]. N4A is

Abbreviations: N4A, N^4-acetylcytidine; IL-1β, interleukin-1β; TLR, Toll-like receptors; NF-κB, nuclear factor κB; NLR, NOD-like receptor; NLRP3, nucleotide-binding domain, leucine-rich repeat containing protein 3; HMGB1, high-mobility group box 1; OD, optical density; RAGEs, receptors for advanced glycation end products; RT-PCR, reverse transcription polymerase chain reaction; ASC, apoptosis-associated speck-like protein containing a CARD

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one biomarker of renal disease in diabetic patients [11] and patients with colorectal cancer [12]. These patients show clear signs of oxidative stress. Under acute or chronic oxidative stress, tRNA is degraded [13]. After N4A is released from tRNA turnover, it cannot be reutilized and further degraded, instead it is excreted in urine. Similarly, elevated oxidative stress is present in the CNS of the majority of elderly people and patients with neurodegenerative diseases such as Alzheimer's disease [14]. Furthermore, other studies also show that inhibiting the *N*-acetyltransferase 10 (NAT10) which catalyzes N4A formation, enhances health span in a mouse model of human accelerated aging syndrome [15] and normalizes the nuclear shape of laminopathic cells [16]. These observations prompted us to investigate if N4A is positively associated with neuroinflammation. Our preliminary data found that N4A was able to activate microglia and induce persistent expression and release of alarmin high mobility group box-1 (HMGB1). Therefore, we hypothesized that N4A might sustain NLRP3 inflammasome activation via induction of HMGB1 expression and release.

To test our hypothesis, we addressed the following questions:1) Does N4A activate microglia? 2) Does N4A induce sustained expression and release of HMGB1? 3) Does N4A activate NFkB signaling and upregulate NLRP3 expression? 4) Does N4A-mediated NFκB signaling and NLRP3 expression require HMGB1 expression and release? 5) Is NLRP3 inflammsome required for N4A-mediated HMGB1 expression? Our findings demonstrated that N4A primed and activated NLRP3 inflammation. N4A-induced HMGB1 expression feedback activates NFκB signaling/NLRP3 expression and NLRP3 inflammasome activation, thus leading to sustained NLRP3 inflammasome activation. Our study provides a novel insight into nucleoside signaling in neuroinflammation.

2. Results

2.1. N4A activates BV2 microglia through $A_{2A}R$ signaling

N4A is a member of nucleoside metabolites. Its chemical structure is shown in Fig. 1A. We first examined whether N4A has cytotoxicity on BV2 microglia. CCK8 assay showed that N4A had no significant growth inhibition on BV2 microglia from the concentration of 0.1 to 2.0 mM at post-24 h treatment, while exhibited significant growth inhibition on BV2 microglia from the concentration of 0.3 mM at post-48 h treatment (Fig. 1B). Currently, we do not have a widely accepted cellular model to test how drug treatment induces chronic inflammation. In addition, as mentioned before, the way to remove N4A from the body is very limited, thus its accumulation during the process of inflamation is highly plausible. Therefore, N4A (0.3 mM and 1.0 mM) were used in the following experiments. N4A was shown to induce pro-IL1β in mouse macrophages, human neutrophils and human THP1 cells [10], suggesting that N4A is able to activate macrophage. To assess whether N4A is able to activate BV2 microglia, we first examined whether N4A may upregulate several markers for the activation of BV2 microglia. Our qPCR analysis revealed that N4A treatment significantly upregulates mRNA levels of markers of primed microglia such as CD86, major histocompatibility complex II-IIA (MHCII-IIA), MHCII-Ii and MHCII-DM (Fig. 1C), indicating that N4A was able to activate BV2 microglia.

Activating Microglia can be accomplished by activation of receptors that signal through nuclear factor-κB (NFκB) pathways such as Toll-like receptors (TLRs), IL-1R, tumor necrosis factor receptor (TNFR). Therefore, we tested whether N4A acts like LPS and signals through TLR4. Unlike LPS, N4A failed to activate TLR4 mRNA (Fig. 1D). In addition, PPARγ is also a receptor for macrophage activation. Therefore, we examined whether N4A mediates BV2 microglia activation via PPARγ. Similarly, N4A was unable to affect PPARγ (Fig. 1E). Since adenosine promotes macrophage activation via A_{2A} receptor ($A_{2A}R$) [17], and $A_{2A}R$ is upregulated in the preclinical stage and in overt Alzheimer's Disease (AD) when compared to controls [18], we hypothesized that N4A might activate microglia via $A_{2A}R$ like adenosine. We first examined if N4A may signal through $A_{2A}R$. As shown in Fig. 1F (left panel), N4A remarkably activates $A_{2A}R$. Since activated microglia may produce NO which is catalyzed by inducible nitric oxide synthase (iNOS), we examined whether $A_{2A}R$ could regulate iNOS expression in N4A-activaed microglia. As expected, iNOS mRNA expression was significantly increased by 14.5-fold after 3 h of exposure to N4A, and this effect was significantly decreased by SCH58261, which is a $A_{2A}R$ competitive antagonist (Fig. 1F, right panel). Collectively, these results indicate that N4A activates BV2 microglia through $A_{2A}R$ signaling.

2.2. N4A induces sustained HMGB1 expression

N4A has been shown to induce IL-1β in human THP1 leukemia cells and macrophages by activating NLPR3 inflammasome [10]. To further evaluate the effect of N4A on inflammatory factors in BV2 microglia, we tested several cytokines. As shown in Fig. 2, N4A induces the expression of IL-1β, Arg-1, iNOS and S100A8 (Fig. 2A), but inhibits tumor necrosis factor-α (TNFα) (Fig. 2B). N4A can induce the secretion of IL-1β, indicating that N4A-mediated production of inflammatory cytokines is dependent on inflammasome activation. However, the molecular mechanism underlying N4A-mediated inflammasome activation in microglia is still unclear.

An increasing evidence demonstrates that HMGB1 , is a risk factor for the progression of neuroinflammation and chronic degeneration by priming neuroinflammation in the brain [19,20]. Therefore, we first examined the role of N4A on HMGB1 expression. Surprisingly, we found that N4A markedly enhances HMGB1 mRNA expression in BV2 microglia at both 3 h and at 6 h (Fig. 2C). We confirmed HMGB1 expression at protein levels. Western Blot analysis showed that N4A-induced HMGB1 levels at 6 h were markedly higher than at 3 h (Fig. 2D). These results suggest that N4A-induced HMGB1 release might feedback to sustain HMGB1 expression.

2.3. N4A activates NFkB signaling and upregulates NLRP3 expression

Since $A_{2A}R$ is associated with neuroinflammation [21] and involved in activating NFkB in rat hippocampus after exposure to 3,4-methylenedioxy-N-methamphetamine (MDMA) [22], we tested whether N4A mediates NFkB signaling. Rel A, a subunit of NFκB, was greatly upregulated at both mRNA level (Fig. 3A) and protein level (Fig. 3B) by N4A treatment at 3 h and 6 h, suggesting that N4A activates NFkB signaling. N4A treatment significantly upregulated NLRP3 expression at both mRNA (Fig. 3C) and protein levels (Fig. 3D). Taken together, these results indicate that N4A activates NFκB signaling and upregulates NLRP3 expression.

2.4. N4A-mediated NFκB signaling and NLRP3 expression requires HMGB1 expression and release

Since HMGB1 activates NLRP3 inflammation via NFkB signaling [23], we examined whether N4A-mediated HMGB1 regulated NFkB signaling and NLRP3 expression. First, we assessed the role of N4A-mediated HMGB1 expression in NFκB signaling and NLRP3 expression. We first inhibited HMGB1 expression using siRNA against HMGB1(siHMGB1) or non-specific scramble RNA(siScr) in BV2 microglia, and then treated these BV2 microglia with N4A. As shown in Fig. 4A, HMGB1 expression was remarkably upregulated by N4A treatment at 0.3 mM or 1.0 mM in the presence of siScrambel (siScr), but siHMGB1 significantly abrogated N4A-induced HMGB1 expression. siHMGB1 also blocked the induction of REL B by N4A. Surprisingly, siHMGB1 not only inhibited the induction of REL A and NLRP3 expression by N4A treatment, but also resulted in the reduction of REL A, REL B and NLRP3 expression, when compared to the control (Fig. 4A), suggesting that basal level of HMGB1 expression might be required to maintain the expression of REL A and NLRP3.

Secondly, we investigated whether N4A-mediated HMGB1 release had similar effect on NFκB signaling and NLRP3 expression. We first

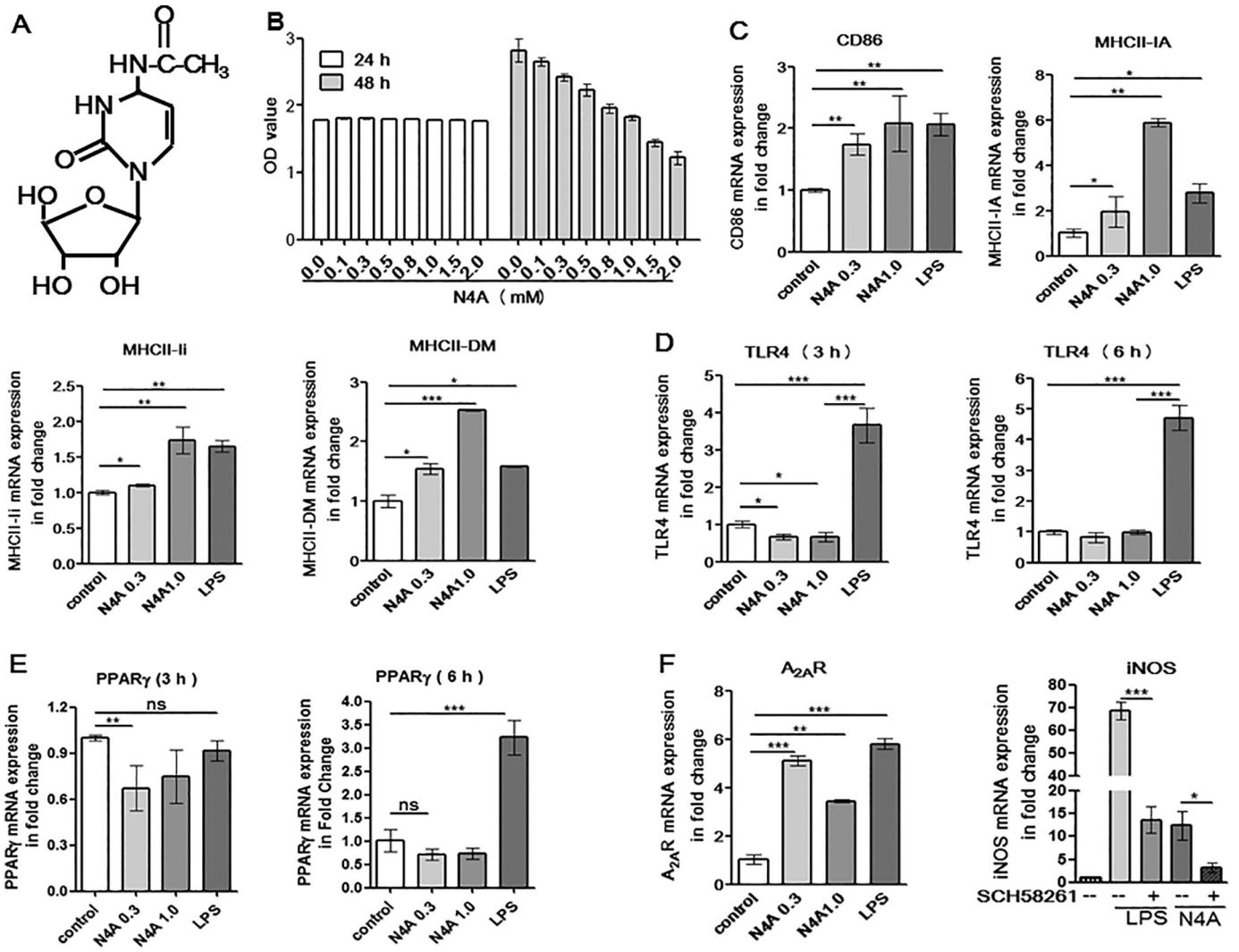

Fig. 1. N4A activates microglia (A) Chemical structure of N4A. (B) The effect of N4A on BV2 microglia growth. BV2 microglia were cultured with indicated concentrations of N4A (0, 0.1, 0.3, 0.5,0.8,1.0, 1.5, 2.0 mM) for 24 h and 48 h, respectively. The cell viability was determined by CCK8 assay. OD represents optical density at 450 nm. The values presented are the means ± SD of three independent experiments. (C) N4A treatment upregulated CD86, MHCII-IA, MHCII-li and MHCII-DM. BV2 microglia were treated with indicated concentration of N4A or 50 ng/ml LPS for 3 h. BV2 microglia were collected for analyzing above gene expression by qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments. (D) N4A failed to activate TLR4. BV2 microglia were treated with LPS (50 ng/mL), N4A at 0.3 mM or 1.0 mM for 3 h and 6 h. TLR4 mRNA expression levels were analyzed by qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments. (E) N4A was unable to signal through PPARγ. BV2 microglia were treated with LPS (50 ng/mL), N4A at 0.3 mM or 1.0 mM for 3 h and 6 h. PPARγ mRNA expression levels were analyzed by qPCR. (F) N4A activates $A_{2A}R$. Left panel: BV2 microglia were treated with LPS (50 ng/mL), N4A at 0.3 mM or 1.0 mM for 3 h. LPS served as a positive control. $A_{2A}R$ mRNA expression levels were analyzed by qPCR. Right panel: BV2 microglia were pretreated with SCH 58261 (50 nM) for 1 h and then challenged with LPS (50 ng/mL) or N4A (0.3 mM) for 3 h. INOS mRNA expression levels were analyzed by qPCR.

tested whether N4A may induce HMGB1 release. Western blotting was used to assess HMGB1 levels released from cell culture supernatants. As expected, N4A treatment induced much stronger release of HMGB1 than LPS, whereas adenine did not (Fig. 4B). To test whether HMGB1 is clinically relevant, we measured plasma HMGB1 levels in AD patients presenting in oxidative stresses (Data not shown) and healthy controls. As depicted in Fig. 4C, ELISA assay showed that plasma HMGB1 levels were significantly higher in AD patients than in healthy controls. We further examined whether N4A-mediated HMGB1 release re-induces NLRP3 and HMGB1 expression. We collected the supernatants from BV2 microglia transfected with siHMGB1 or siScr. for 48 h, and then treated BV2 microglia with the supernatants. siScr supernatant remarkably upregulated the expression of HMGB1 and NLRP3, but the siHMGB1 supernatants did not (Fig. 4D). Taken together, our results indicate that N4A-induced HMGB1 expression and its release is required for NFkB signaling, NLRP3 expression and persistent HMGB1 expression.

2.5. NLRP3 inflammsome is required for N4A-mediated HMGB1 expression

NLRP3 inflammasome has been shown to mediate HMGB1 release [24,25]. To investigate whether NLRP3 inflammasome is required for N4A-mediated HMGB1 expression, we first inhibited NLPR3 expression by RNAi (Fig. 5A, left panel). N4A-induced HMGB1 expression (6 h) was abrogated by NLRP3 silencing (Fig. 5A, right panel), suggesting that persistent HMGB1 expression requires NLRP3 expression. Secondly, we used RAW 264.7 cell line, a mouse macrophage, which naturally lacks NLRP3 inflammasome adaptor named apoptosis-associated speck-like protein containing a CARD (ASC). As shown in Fig. 5B, N4A treatment significantly upregulated REL B (Fig. 5C), HMGB1

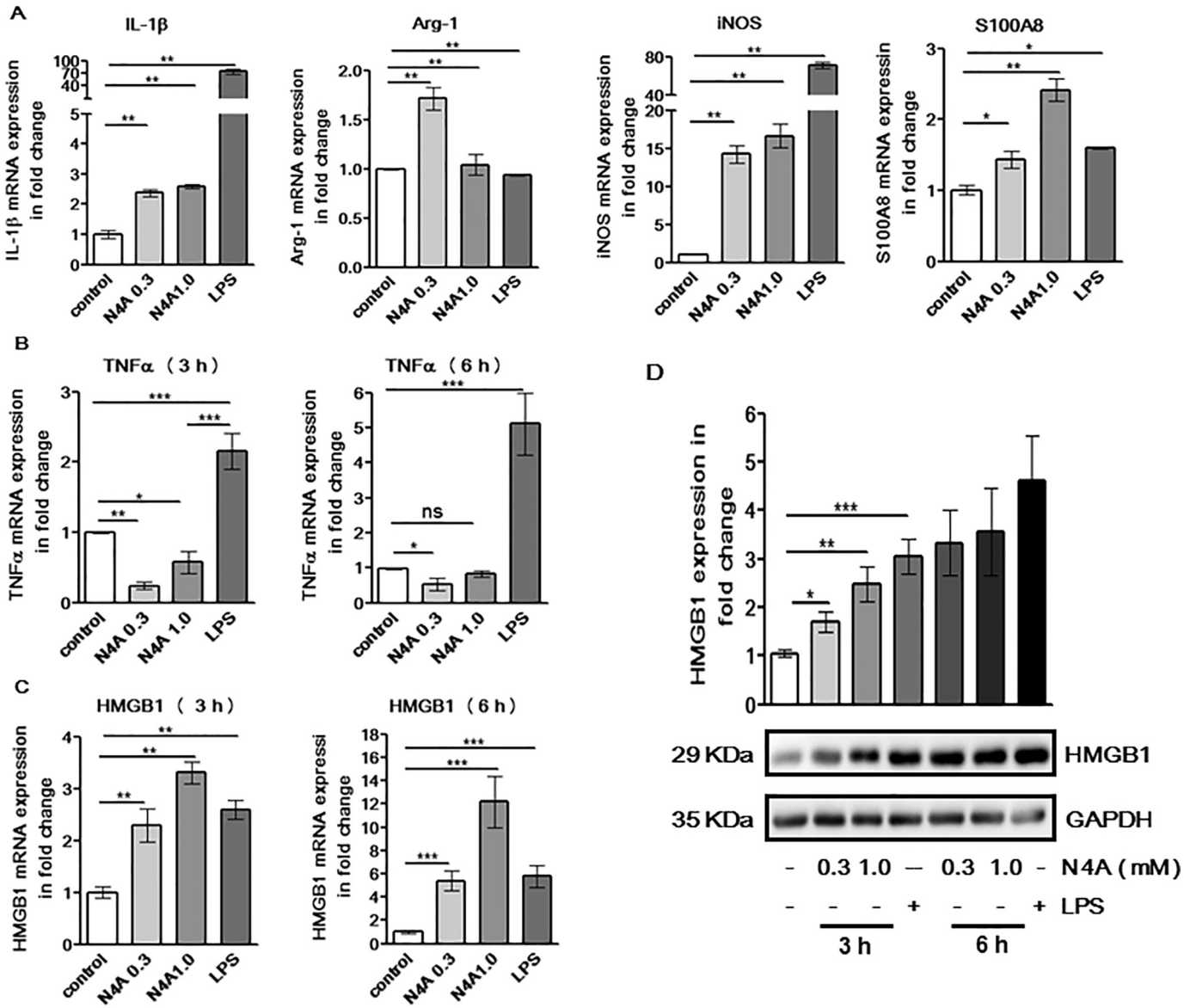

Fig. 2. The effect of N4A on the production of inflammatory cytokines (A) The induction of inflammatory cytokines by N4A. BV2 microglia were treated with indicated concentration of N4A or 50 ng/ml LPS for 3 h. Indicated gene expression levels were assessed by qPCR. (B, C) BV2 microglia were treated with indicated concentration of N4A or 50 ng/ml LPS for 3 h and 6 h. The mRNA expression levels of TNFα and HMGB1 were assessed by qPCR. (D) N4A induces persistent HMGB1 expression. BV2 microglia were treated with N4A (0.3 mM, 1.0 mM), or LPS (50 ng/mL) at final concentration for 3 h and 6 h, respectively. HMGB1 expression in cell lysates was assessed by Western blot analysis. GAPDH served as a loading control. Bar chart shows quantification of protein levels normalized to GAPDH in each sample. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments.

(Fig. 5D) and NLRP3 (Fig. 5E) in BV2 microglia, but not in RAW264.7 cells, suggesting that NLRP3 inflammasome is essential for N4A-mediated HMGB1 expression. Although LPS induced REL B and NLRP3 expression in RAW264.7 cells, but did not induced HMGB1 expression, suggesting that HMGB1 expression might require successful assembly of NLRP3 inflammasome. Unlike N4A, adenine upregulated both REL B and HMGB1 expression, but not NLRP3 expression. Taken together, these results indicate that sustained N4A-mediated HMGB1 expression is dependent on NLPR3 inflammasome.

3. Discussion

Persistent NLRP3 inflammasome activation contributes to chronic low-grade inflammasome activation which leads to the pathogenesis of aging, AD and PD. Activation of the NLRP3 inflammasome requires priming signal and then NLRP3-specific activating signal [26,27]. Once inflammasome activation is initiated, macrophages are tolerated to subsequent initiating signals [27], indicating that persistent NLRP3 inflammasome activation requires additional stimuli other than the initiating signal. However, it is unclear how inflammasome activation is sustained in chronic inflammation, repair and fibrosis [28,29]. In this study, we demonstrated that N4A regulated sustained NLRP3 inflammasome via HMGB1 signaling in microglia. N4A may prime NLRP3 inflammasome by acting as signal 1 to induce NLRP3 and HMGB1 expression, and then N4A-induced HMGB1 acted as signal 2 to promote the assembly of NLRP3 inflammasome and subsequent activation. These results are consistent with a previous finding that inflammasome activity is sustained by adenosine via $A_{2A}R$ signaling after initial activation [9].

We first found that N4A was able to activate microglia without

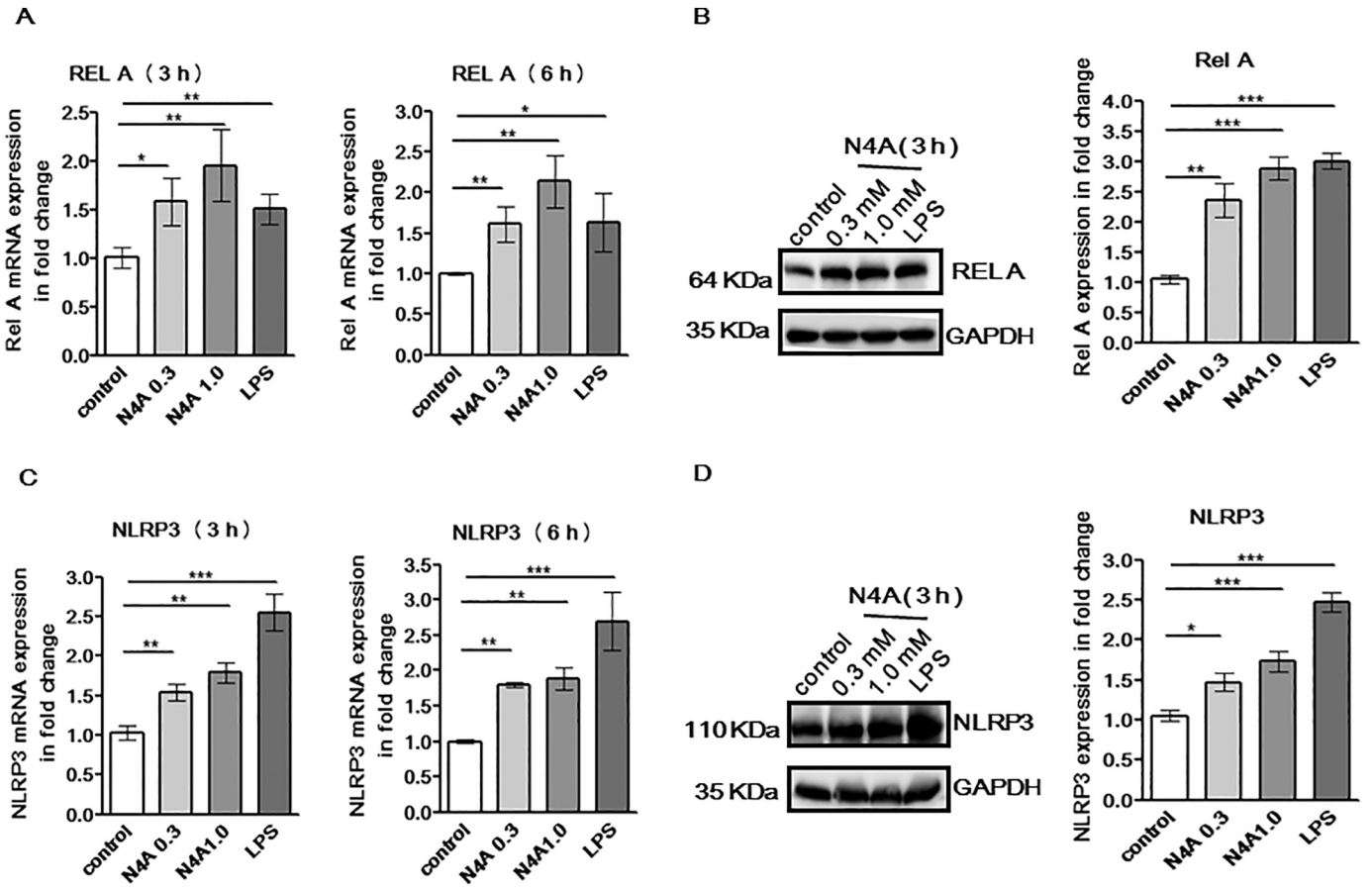

Fig. 3. N4A upregulated NFkB and NLRP3 expression BV2 microglia were treated with LPS (50 ng/mL), N4A at 0.3 mM or 1.0 mM for 3 h or 6 h. mRNA expression levels of indicated genes were assessed by qPCR and protein expression by Western Blot. (A, B) N4A activated NFκB signaling. (C, D) N4A induced NLRP3 expression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments.

liposaccharide priming. N4A upregulated the expression levels of microglial CD86, MHCII-IIA, MHCII-Ii and MHCII-DM. This is in agreement with a recent study which showed that N4A was able to prime and activate NLRP3 and NLRC4 inflammasomes to produce IL-1β [10]. In addition to produce IL-1β, we found that N4A-activated microglia produced and released significantly elevated HMGB1 when compared to control. Moreover, N4A also inhibited TNFα expression, consistent with a previous report that adenosine inhibits TNFα expression and release from mouse peritoneal macrophages via A2AR [17].

Our results showed that N4A-mediated HMGB1 release is required for persistent HMGB1 expression through NFκB signaling and upregulating NLRP3 expression. First, inhibition of HMGB1 expression by RNAi abolished N4A-mediated upregulation of HMGB1, NFκB subunits and NLRP3 expression levels as well as HMGB1 release. Secondly, the supernatant derived from BV2 microglia transfected with specific siRNA against HMGB1 abolished N4A-upregulated NFκB signaling and NLRP3 expression. These results suggest that N4A-mediated HMGB1 expression and feedback release regulates NFκB signaling and NLRP3 expression. Our findings are supported by other study showing that stress-induced HMGB1 acted as a priming stimulus of microglia and the NLRP3 Inflammasome in the hippocampus of male Sprague Dawley rats [30]. Our results are also in agreement with previous studies showing that HMGB1 upregulates NFκB expression [31] and NLRP3 expression in vascular smooth muscle cells (VSMCs) [32] and activates NLRP3 inflammasome in response to acutely elevated intraocular pressure during acute glaucoma development [23]. Collectively, our results suggest that N4A is able to act as signal 1 to prime NLRP3 inflammasome.

We demonstrated that N4A-mediated HMGB1 release was dependent on NLRP3 inflammasome. First, silencing NLRP3 expression abrogated N4A-mediated upregulation of HMGB1 expression and release, suggesting that NLRP3 inflammasome-mediated HMGB1 release is required for sustained HMGB1 expression by N4A. Secondly, adenine was able to upregulate HMGB1 expression, but it failed to induce NLRP3 expression and HMGB1 release, indicating that microglial HMGB1 release was NLRP3-dependent. Similarly, N4A failed to induce both HMGB1 and NLRP3 expression in mouse macrophage cell line RAW264.7 cells due to the lack of ASC, indicating that N4A-mediated HMGB1 expression and release requires NLPR3 inflammsome activation and vice versa. Furthermore, LPS induced REL B and NLRP3 expression in RAW264.7 cells, but not upregulation of HMGB1 and subsequent release. These results are in agreement with other authors who showed that NLRP3 inflammasome mediates HMGB1 release [24,25]. Taken together, our results indicate that sustained N4A-mediated HMGB1 expression is dependent on NLPR3 inflammasome.

One limitation of this study is that our experiments have been conducted on cell lines and human plasma. In the future, we will expand our experiments to primary microglia and mice. We will also investigate molecular mechanisms underlying N4A in HMGB1 induction. In summary, our study reported that N4A contributed to persistent production of HMGB1 via HMGB1/NLRP3 inflammasome in microglia. Since HMGB1 has a detrimental effect in the pathogenesis of various neurological diseases, it is critical to identify inducers of HMGB1 expression. Our results provide an insight into NLRP3 inflammsome activation by an endogenous nucleotide metabolite via HMGB1 signaling in microglia.

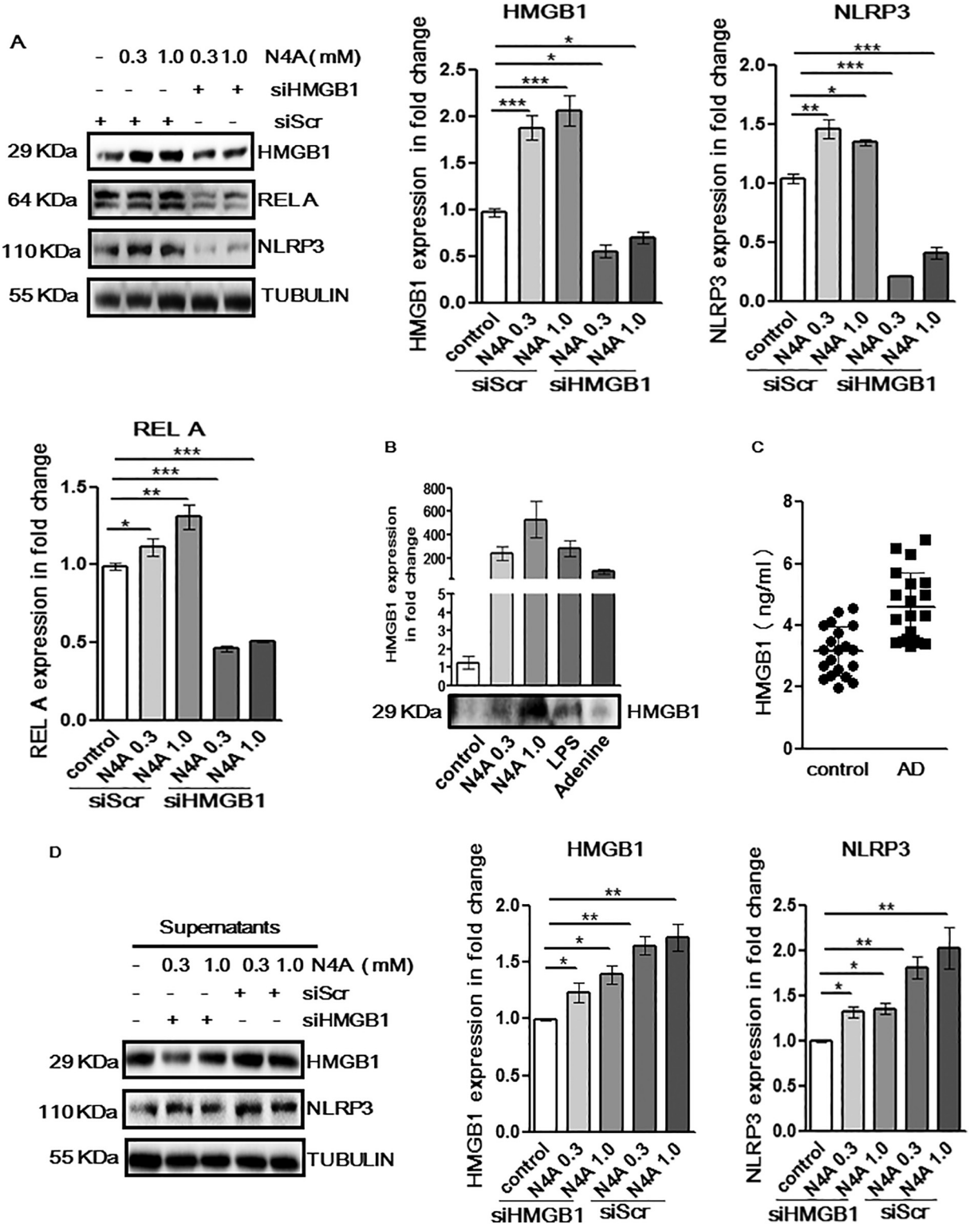

Fig. 4. HMGB1 expression and release is required for NFκB signaling and NLRP3 expression (A) The effect of HMGB1 expression on NFκB signaling and NLRP3 expression. BV2 microglia were first transfected with siHMGB1 or siScr for 48 h, then treated with N4A (0.3 mM, 1.0 mM) at final concentration for 3 h. HMGB1, REL A and NLRP3 expression were assessed by Western Blot. TUBULIN served as a loading control. (B) The effect of N4A on HMGB1 release. N4A induces HMGB1 release. BV2 microglia were treated with N4A (0.3 mM, 1.0 mM), or LPS (50 ng/mL) , or adenine (0.3 mM) at final concentration for 3 h, and then supernatants were collected and further centrifuged to remove cell debris. HMGB1 release in supernatants was assessed by Western blot analysis. (C) Plasma HMGB1 expression levels in patients with Alzheimer's disease and in healthy controls. Plasma HMGB1 was determined by human HMGB1 ELISA kit. (D) The supernatants derived from N4A treatment upregulated HMGB1 and NLRP3 expression. BV2 microglia were first transfected with siHMGB1 or siScr for 48 h, then treated with N4A (0.3 mM, 1.0 mM), or LPS (50 ng/mL) at final concentration for 3 h. Supernatants were added to naïve BV2 microglia for 6 h. HMGB1 and NLRP3 expression were assessed by Western Blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments.

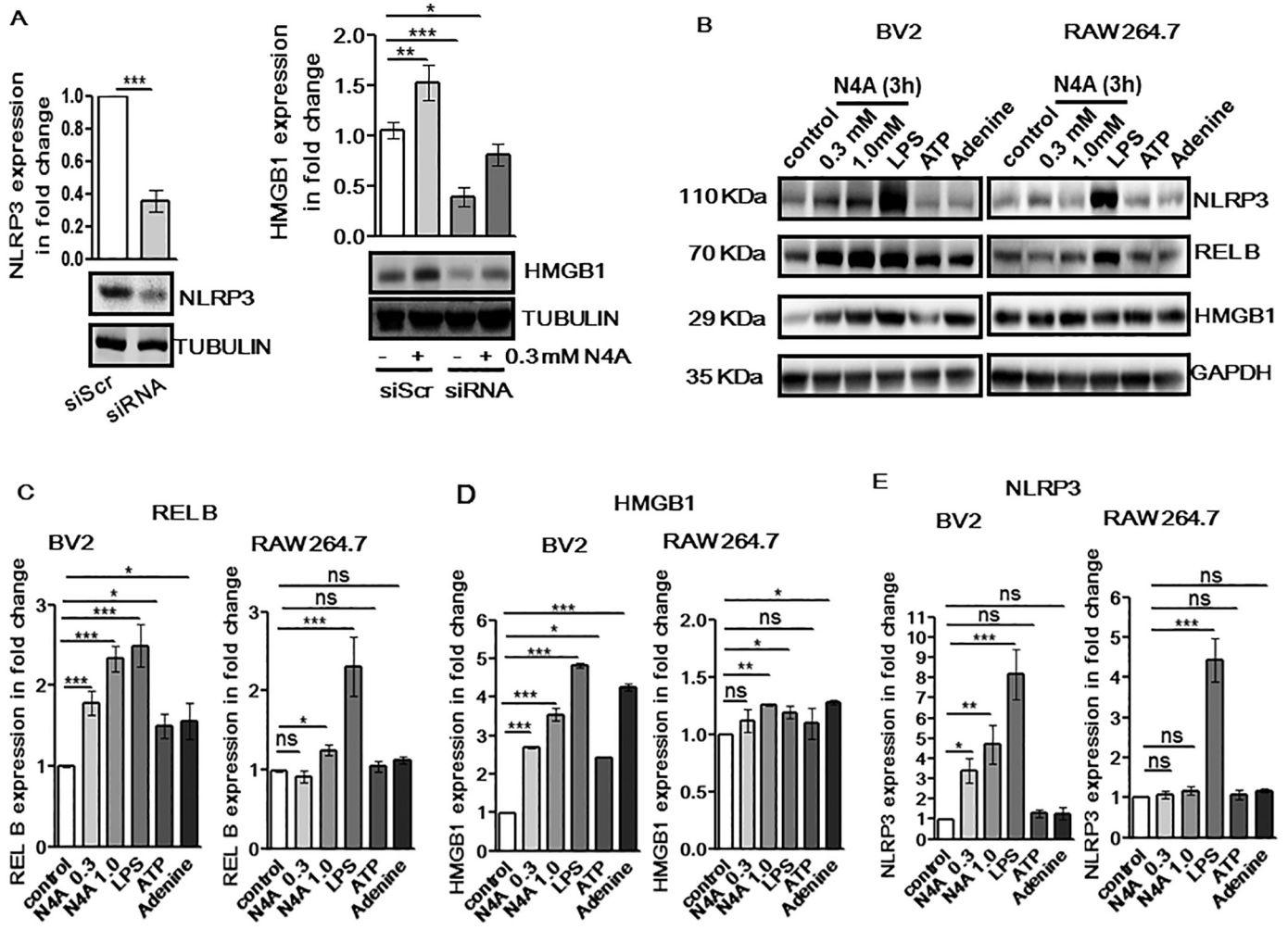

Fig. 5. NLRP3 inflammsome regulates N4A-mediated HMGB1 expression (A) The effect of NLRP3 expression on N4A-mediated HMGB1 expression. Left panel: BV2 microglia was transfected with siRNA against NLRP3(siNLRP3) or scramble (siScr). The efficiency of NLRP3 silencing was assessed by Western blot analysis. Right panel: after transfection with siNLRP3 or siScr for 48 h, BV2 microglia was treated with 0.3 mM N4A for 3 h. HMGB1 expression levels were assayed by Western Blot. (B) BV2 microglia or RAW264.7 cells were treated with N4A (0.3 mM or 1 mM), LPS (50 ng/mL), ATP (4 mM) or adenine (0.3 mM) for 3 h, respectively. Cell lysates were analyzed by Western blot analysis. (C) REL B expression between in BV2 microglia and RAW264.7 cells. (D) HMGB1 expression between in BV2 microglia and RAW264.7 cells. (E) NLRP3 expression between in BV2 microglia and RAW264.7 cells. $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. Control group: cells treated with DMSO. The values presented are the means ± SD of three independent experiments.

4. Materials and methods

4.1. BV2 microglia and RAW264.7 cell culture

BV2 murine microglia were purchased from China Infrastructure of Cell Line Resources (Beijing, China) and RAW254.7 murine macrophage cell line was purchased from ATCC. Both BV2 microglia and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% heat-inactivated fetal bovine plasma (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. The reagents used in this study included: Lipopolysaccharide (LPS, Sigma), N4A(Shanghai Macklin Biochemical Co.,Ltd), ATP (A1852, Sigma) and adenine (A8330, Sigma), SCH 58261(MedChemExpress).

4.2. Cell count kit (CCK8) assay

BV2 were seeded in 5000 microglia/well in 96-well plate. On the second day, BV2 microglia were treated with N4A at final concentrations (0, 0.1, 0.3, 1.0, 1.5 mM) for 24 h and 48 h. The cell viability was determined by CCK8 assay kit (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, P. R. China) following the instructions.

4.3. Reverse transcription and real quantitative real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen). After reverse transcription reactions were performed with the PrimeScript™ RT Master Mix cDNA Synthesis Kit (Takara Bio) to get cDNA. Samples were analyzed using pairs of primers specific for genes of interest and β-actin mRNAs. Sequence-specific amplification was detected by fluorescent signal of SYBR Green (Takara Bio) by using Real-time PCR Detector (Applied Biosystems, Australia). Relative gene expression was determined using the 2-ΔΔCT method normalized to the internal control gene expression. The sequences of PCR primers used are listed in Table 1. β-actin was used as the internal control gene.

4.4. Western blot analysis

Cells were lysed in pre-chilled lysis buffer (20 mM HEPES, 150 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20% glycerol, 1% Triton X-100) with protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN) for 30 min at 4 °C, then centrifuged at 14,000 ×*g* for 20 min at 4 °C. The concentration of cell lysates was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Cell lysates were subjected to 12% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF)

Table 1
Primer sequences used in this study.

Gene	Forward primer	Reverse primer
CD86	5′-TCAATGGGACTGCATATCTGCC -3′	5′-CAGCTCACTCAGGCTTATGTTTT-3′
MHCII-Ii	5′-GACCATCACCTCCCAGAACC-3′	5′-TGGGTCATGTTGCCGTACTT-3′
MHCII-I-A^{β}	5′-AGCAGGTGTGAGTCCTGGTG-3′	5′-TAGCACTCGCCCTTGAACTG-3′
MHCII-H2-DM	5′-GTCGGTGGAAAGAGGTTGCT-3′	5′-CCTCTGCCATTGCATTTGAC-3′
TLR4	5′-GCAGAAAATGCCAGGATGATG-3′	5′-CTACCTCTATGCAGGGATTCAA-3′
TNFα	5′-CATCTTCTCAAAATTCGAGTGAC-3′	5′-TGGGAGTAGACACAAGGTACAA-3′
HMGB1	5′-CCGGGAGGAGCACAAGAAGA-3′	5′-CCCTTTTTCGCTGCATCAGG-3′
S100A8	5′- AAATCACCATGCCCTCTACAAG −3′	5′-CCCACTTTTATCACCATCGCAA −3′
Rel A	5′-TCATGAAGAAGAGTCCTTTCA-3′	5′-CTGGCTTGGGGACAGGAG-3′
IL-1β	5′- CCTGCAGCTGGAGAGTGTGGA-3′	5′-TGTGCTCTGCTTGTGAGGTGCT-3′
β-actin	5′-GGTCATCACTATTGGCAACG-3′	5′-ACGGATGTCAACGTCACACT-3′
Nlrp3	5′-GTGGTGACCCTCTGTGAGGT-3′	5′-TCTTCCTGGAGCGCTTCTAA-3′
iNOS	5′-GGAGTGACGGCAAACATGACT-3′	5′-TCGATGCACAACTGGGTGAAC-3′
PPARγ	5′- GTGCCAGTTTCGATCCGTAGA −3′	5′- GGCCAGCATCGTGTAGATGA −3′

membranes. PVDF Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated overnight at 4 °C with the indicated primary antibodies: rabbit anti-HMGB1(Cell Signaling, 6893), rabbit anti-NLRP3 (Cell Signaling, 15,101), rabbit anti-RELB (Cell Signaling , 10,544), rabbit anti-NF-KB (P65) (Abcam , ab16502), anti-rabbit GAPDH (14C10) (Cell Signaling,), β-Tubulin (9F3) Rabbit mAb ((Cell Signaling , 2128). After washing, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit (1:1000; Cell Signaling), or anti-mouse (GE Healthcare) antibody for 1 h at room temperature, then the protein bands were detected by an enhanced chemiluminescence detection system (ECL kit; Minipore), and the signals were visualized using Licor Odyssey Scanner. Signal intensity was quantified using Image J.

4.5. Human plasma

Plasma were collected from twenty AD patients and twenty controls in the Third affiliated Hospital of Guizhou Medical University (GMU) in China. AD patients were clinically diagnosed in Department of Neurology and Department of Psychiatry and Department of Medical Examination. The controls without dementia or MCI were gathered from Department of Medical Examination. This study was approved by the Institutional Ethics Committees of the Third affiliated Hospital of GMU. Informed consent for participation was obtained either directly or from a legal guardian.

4.6. Measurement of plasma HMGB1

Plasma human HMGB1 levels were measured using human high mobility group box 1 (HMGB1) ELISA kit (CSB-E08223h, Cusabio Biotech Co. LTD). Following the instructions, the optical density of each well were determined using a microplate reader set to 450 nm.

4.7. Small interfering RNA (siRNA) transfection

BV2 cells were seeded on 6-well plates (3×10^5 cells/well) in complete DMEM (without antibiotics) for overnight and transfected with 50 nM siRNA targeted to HMGB1 mRNA or with 50 nM scramble siRNA (siScr, as negative control), using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The siRNA sequences are as follows: siHMGB1, 5′- GGUAACAUUUUCAUCCAU ATT-3′, antisense: 5′–UAUGG- AUGAAAAUGUUACCCT-3′; siSramble (siScr), 5′-UUCUCCGAACGUGUCA- CGUdTdT-3′, 5′- ACGUGACACGU UCGGAGAAdTdT-3′; siNLRP3, 5′-CGGCCUUACUUCAAUCUGUTT-3′ and 5′-ACAGAUUGAAGUAAGGCCGTT-3′; siScr, 5′-UUCUCCGAACGU GUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′. Knockdown efficiency was checked by Western blotting.

5. Statistical analyses

The data are represented as the means ± SD. The statistical analyses were performed with one-way ANOVA plus Bonferroni post-hoc test for multiple treatment groups. The P values are indicated in the figures with asterisks: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Data were analyzed using GraphPad Prism software (GraphPadprism, version 5.01, GraphPad Software, Inc., San Diego, CA, USA).

Author contributions

H.B., W.F·Y and Q.F.Z conceived and designed experiments. J.J.D and Q.F.Z performed experiments. D.Q.L, J.J.D and Q.F.Z analyzed data and interpreted results. DJY and X.H·H performed experiments and analyzed data. The paper was written by Q.F.Z. All authors read and approved the final version of the manuscript.

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Declaration of interest

The authors declare no competing financial interest.

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