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Original Research

Betamethasone Dipropionate Inhibits NLRP3 Inflammasome Activation by Suppressing Pro-IL-1β Expression

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Abstract

Background: The NOD-like receptor Family, pyrin domain-containing 3 protein (NLRP3) inflammasome is a macromolecular complex critical for inflammatory responses. Its excessive activation or improper regulation is intimately associated with the development of various inflammatory diseases. However, currently available drugs directly targeting the NLRP3 inflammasome are limited. In preliminary analyses, detecting the level of secreted interleukin-1 beta (IL-1β) revealed that betamethasone-17,21-dipropionate (also known as betamethasone dipropionate, BD), a clinically used glucocorticoid, potentially inhibits NLRP3 inflammasome activation. **Methods:** *In vitro*, the role and preliminary mechanism of BD in inhibiting the activation of NLRP3 inflammasome were investigated in THP-1-differentiated macrophages and bone marrow-derived macrophages (BMDMs) using enzyme-linked immunosorbent assay (ELISA), Cell Counting Kit-8 (CCK-8), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and western blotting (WB). The preliminary effects of BD on the assembly of NLRP3 inflammasome were assessed in HEK293T cells overexpressing NLRP3/ASC/caspase-1/NEK7 through drug affinity responsive target stability (DARTS) and co-immunoprecipitation (Co-IP) approaches. *In vivo*, the effect of BD on LPS-induced systemic inflammation was assessed by measuring serum concentrations of IL-1β and TNF-α via ELISA, and by recording mouse survival rates and body weights. **Results:** BD significantly inhibited NLRP3 inflammasome activation by suppressing pro-IL-1β expression *in vitro*. Mechanistic studies showed that it decreased pro-IL-1β expression by suppressing NF-κB signaling. *In vivo*, BD downregulated the serum concentrations of IL-1β and TNF-α, and increase the survival rate of mice using the LPS-induced systemic inflammation model. **Conclusions:** Collectively, our data verify that BD inhibits NLRP3 inflammasome activation by suppressing pro-IL-1β expression. These findings suggest that BD may be a potential therapeutic approach for inflammatory diseases. However, further studies are needed to elucidate its precise role and specific mechanism in clinical practice.

Keywords: inflammation; betamethasone-17,21-dipropionate; inflammasomes; NLR Family, Pyrin Domain-Containing 3 Protein; pyroptosis; interleukin-1 beta

1. Introduction

The NLRP3 inflammasome has been extensively studied in the past two decades, functioning as a key sensor for danger- and damage-related signals and as an essential mediator of host defenses against pathogen infection [1]. Among the molecular patterns it can recognize are pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), such as nigericin and aluminum adjuvant [2,3,4]. However, overactivation or dysregulation of NLRP3 inflammasome has been strongly associated with the onset of multiple inflammatory diseases, such as sepsis, atherosclerosis, neurodegenerative diseases, and various autoimmune and metabolic disorders [5,6,7,8,9,10,11,12].

Canonical NLRP3 inflammasome activation depends on a two-signal system consisting of distinct priming and activation triggers [13,14]. The priming signal is driven by Toll-like receptor 4, which recognizes lipopolysaccharide (LPS) and activates nuclear factor-κB (NF-κB) signaling pathway, thereby elevating pro-IL-1β and NLRP3 expression [13]. Thus, the priming signal provides the essential components required for the activation signal to proceed. The activation signal stems from PAMP- or DAMP-driven oligomerization of NLRP3, which then serves as a molecular scaffold, enabling the assembly of adaptor protein ASC and effector protein caspase-1 (Casp-1) into a signaling platform, thereby triggering Casp-1 activation [15,16]. Finally, activated Casp-1 proteolytically processes pro-IL-1β and gasdermin D (GSDMD), resulting in mature IL-1β secretion and GSDMD-mediated pyroptosis [17,18]. Several mechanisms, including K^+ efflux, mitochondrial dysfunction, and Ca^{2+} influx, can facilitate NLRP3 inflammasome assembly [19,20]. Never in mitosis A (NIMA)-related ki-

nase 7 (NEK7) also directly engages NLRP3, thereby dictating the assembly of NLRP3 inflammasome [21,22].

Over recent years, several inhibitory compounds have been demonstrated to suppress NLRP3 inflammasome activation via diverse mechanisms. MCC950, oridonin, and britannin have been identified as direct small molecule inhibitors targeting NLRP3 [23,24,25]. These compounds can specifically bind to the NLRP3 protein and suppress its conformational transition via covalent interaction, thereby inhibiting NLRP3 inflammasome activation. Entrectinib and berberine, in contrast, can directly target NEK7 to disrupt the NLRP3-NEK7 interaction [26,27], while necrosulfonamide and disulfiram can directly target GSDMD to reduce GSDMD-mediated pyroptosis and inhibit mature IL-1β secretion [28,29]. Loteprednol etabonate and ginsenoside Rg3 can suppress pro-IL-1β expression, ultimately restricting NLRP3 inflammasome activation [30,31]. At present, very few drugs targeting NLRP3 inflammasome are clinically available. As such, there is a clear need to identify new inhibitors capable of targeting this inflammasome complex to aid the treatment of inflammatory diseases.

In the previous study, we screened 144 FDA-approved or clinically used drugs and determined that loteprednol etabonate exhibits an inhibitory effect on NLRP3 inflammasome [30]. By detecting the level of secreted IL-1β, the data demonstrated that BD potentially suppresses NLRP3 inflammasome activation. BD has been routinely used for treating inflammatory skin diseases, including psoriasis [32]. It has been shown to bind to specific corticosteroid receptors, thereby negatively regulating the production of myriad inflammatory mediators [33]. In the present study, we expanded on these prior bindings, revealing that BD markedly inhibits NLRP3 inflammasome activation by suppressing pro-IL-1β expression, thereby decreasing mature IL-1β secretion and suppressing GSDMD-mediated pyroptosis. Further studies demonstrated that BD attenuates pro-IL-1β expression via blocking the NF-κB signaling cascade, while it may exert no direct influence on the assembly of NLRP3 inflammasome. Moreover, BD can alleviate LPS-induced systemic inflammation, exhibiting remarkable therapeutic effects and improving the survival of the treated mice. Together, the data presented herein demonstrate that BD suppresses NLRP3 inflammasome activation, supporting its utility as a promising intervention for inflammatory disorders.

2. Materials and Methods

2.1 Reagents and Cells

BD (S1688, Selleck Chemicals, Houston, TX, USA); DMSO (D2650, Sigma-Aldrich, St. Louis, MO, USA); nigericin (tlrl-nig, InvivoGen, San Diego, CA, USA); LPS (L2630, Sigma-Aldrich); Phorbol 12-Myristate 13-Acetate (PMA) (P8139, Sigma-Aldrich); Lipofectamine 2000 (11668027, Invitrogen, Carlsbad, CA, USA); anti-GAPDH (1:5000, AC002, ABclonal, Wuhan, Hubei, China); anti-NLRP3 (1:1000, 15101, Cell Signaling Technology (CST), Danvers, MA, USA); anti-IL-1β (1:1000, 12703/12242, CST); anti-caspase-1 (1:1000, 24232/3866, CST); anti-Phospho-p65 (1:1000, 3033, CST); anti-p65 (1:1000, 8242, CST); anti-GSDMD (1:1000, ab209845, Abcam, Cambridge, UK); anti-Flag (1:2000, F3165, Sigma-Aldrich); and anti-HA (1:3000, M180-3, MBL, Nagoya, Aichi, Japan) were purchased for use in this study.

HEK293T (CL-0005, Procell, Wuhan, Hubei, China), THP-1 (CL-0233, Procell), and L929 (CL-0137, Procell) cells were cultured in DMEM, RPMI-1640, and MEM, respectively. All these media, purchased from Gibco (Waltham, MA, USA), were supplemented with 10% fetal bovine serum (FBS) (FSP500, ExCell Bio, Shanghai, China) and 1% penicillin-streptomycin (15140148, Gibco, USA) to culture the relevant cells at 37 °C in a humidified incubator with 5% CO_2. Cells at passage 3 were used for all experiments. All cell lines were validated by STR profiling and tested negative for mycoplasma. C57BL/6 mice of 7–8 weeks of age served as the source for bone marrow, from which BMDMs were collected. Briefly, mice were euthanized by cervical dislocation. Bilateral femurs and tibias were then collected in a sterile environment and immersed in DMEM. A 1 mL syringe was next utilized to aspirate the medium and flush the bone marrow from the bones into a culture dish. Red blood cell lysis buffer was used to remove erythrocytes from the isolated bone marrow cells. Cultivated subsequently in DMEM with 20% L929 cell-conditioned medium were the resultant cells. Macrophage differentiation was achieved after 5–6 days of continuous culture. We validated the identity of primary BMDMs from C57BL/6 mice by detecting the surface marker $CD11b^+$ using flow cytometry, with L929 cells used as a control. We also performed PCR-based DNA electrophoresis and confirmed that the BMDMs were negative for mycoplasma contamination. These results have been added to the "**Supplementary Material**" section.

2.2 Inflammasome Stimulation

THP-1 cells were induced to differentiate using PMA (50 ng/mL). Following 12 h of incubation, the medium was replaced and the cells were cultured for an additional 24 h. After a 12-hour treatment with BD, the supernatant was discarded and replaced with fresh culture medium, followed by the addition of LPS (100 ng/mL) for 6 h. Prior to sample collection, nigericin (10 μM, 1 h) or alum (300 μg/mL, 8 h) was added to activate NLRP3 inflammasome. Similarly, BMDMs were cultured for five days, prior to exposure to BD for 1 h, and then sequentially challenged with LPS (100 ng/mL, 6h) and then with ATP (5 mM, 30 min) or nigericin (10 μM, 1 h). In AIM2/NLRC4 inflammasome activation experiments using BMDMs, BD and LPS treatments were performed as described above, followed by poly (dA:dT) (1 μg/mL, 4 h)/flagellin (1 μg/mL, 4 h) using Lipofectamine

2000. To examine whether RU486 (S2606, Selleck Chemicals, Houston, TX, USA) affects the efficacy of BD, cells received RU486 (1 or 10 μM) as a 15-minute preliminary intervention prior to being treated with BD together with the corresponding stimuli.

2.3 Cytotoxicity Assay

A CCK-8 assay (C0038, Beyotime, Shanghai, China) was used to assess cytotoxicity. Briefly, THP-1 cells and BMDMs were plated in appropriate culture plates in advance, followed by treatment with BD at specified concentrations for 24 h. Next, CCK-8 working solution was introduced into every single pore, followed by 2 hours of incubation in a regular cell incubator before absorbance detection.

2.4 ELISA

ELISA was used to quantify levels of inflammatory cytokines in cell culture supernatants and murine serum based on the directions provided with commercial kits, including an IL-1β ELISA kit (88-7013A, Invitrogen, Carlsbad, CA, USA; 432616/437016, BioLegend, San Diego, CA, USA) and a TNF-α (tumor necrosis factor-α) ELISA kit (430904, BioLegend). Finally, the absorbance of each well was detected using a microplate reader (Multiskan FC, ThermoFisher, Waltham, MA, USA).

2.5 RT-qPCR

RT-qPCR was performed as detailed in a previous study [34]. All primers were generated using the online NCBI platform, and qPCR was determined by the equipment (CFX Connect™, Bio-Rad, Hercules, CA, USA). The primers were as follows:

h*GAPDH*-F/R: *AAGGCTGTGGGCAAGG/TGGAGGAGTGGGTGTCG.*

h*IL-1β*-F/R: *CACGATGCACCTGTACGATCA/GTTGCTCCATATCCTGTCCCT.*

m*GAPDH*-F/R: *TTCACCACCATGGAGAAGGC/GGCATCGACTGTGGTCATGA.*

m*IL-1β*-F/R: *ACCTTCCAGGATGAGGACATGA/AACGTCACACACCAGCAGGTTA.*

2.6 Co-IP and WB

After rinsing with pre-chilled 1× PBS, the cells were lysed using RIPA buffer supplemented with a protease inhibitor mixture under shaking conditions (4 °C, 20 min). Next, the prepared lysates were collected and centrifuged (4 °C, 15 min). Part of the cell lysis product was set aside as the control group, with the rest incubated overnight with an appropriate primary antibody at 4 °C. Afterwards, the reaction mixture was combined with Protein A/G magnetic beads (Thermo Scientific, 88803) (4 °C, 1–4 h). Following incubation, the bead complexes were washed six occasions using RIPA washing buffer. Finally, the beads were combined with 60 μL of 2× SDS (sodium dodecyl sulfate) sample buffer prepared for detection. For WB, protein samples were first separated on 10% SDS-polyacrylamide gels, and followed by transfer onto membranes. Finally, the membranes were visualized using an imaging system (Chemi-Doc™, Bio-Rad, USA). For further details on this approach, see a previously published study [35].

2.7 DARTS

HEK293T cells were transfected with plasmids in a 6-cm culture dish. Afterwards, lysis of cell samples was performed in 1 mL of RIPA buffer following collection. The lysates were split into two equal fractions: one fraction received 50 μM BD, while the other was administered the same volume of DMSO as a vehicle control. Each lysate fraction was preserved at 37 °C over 60 min, after which pronase (10165921001, Roche, Basel, CH, Switzerland) was supplemented to 20 μg/mL, and the mixed solution was held at 37 °C over 4 min. Thereafter, 5× SDS loading buffer was combined with the prepared samples, which were then heat-denatured before Western blotting analysis.

2.8 Establishment of LPS-induced Systemic Inflammation

Briefly, the procedure was performed as described below. 17-20g female C57BL/6 mice (7 to 8 weeks old) were provided by Shulaibao Biotechnology (Wuhan, China). Before the experiments, mice were housed in plastic cages under a 12-hour light/dark cycle, with free access to food and water. Animals that died accidentally, suffered severe injury, or had unrelated complications during the experiment were excluded. Mice were randomly divided into groups. Mice were given BD via intraperitoneal (i.p.) injection, and then were administered LPS at 20 mg/kg via the same route. Blood samples were collected 4 h after LPS challenge, after which the mice were euthanized by cervical dislocation. ELISA was used to measure the serum levels of TNF-α and IL-1β. For preventive administration, mice received i.p. injection of BD, followed by administration of LPS (15 mg/kg). Mice were observed over a 72-hour period, recording both survival rate and body weight. After the recording ended, the mice were euthanized by cervical dislocation. For therapeutic administration, LPS (15 mg/kg) was first injected intraperitoneally into mice, followed 1 h later by BD treatment. The mice were subsequently observed for 96 h, documenting body weight and survival rate at regular intervals. After the observation period ended, the mice were euthanized by cervical dislocation.

2.9 Statistical Analyses

GraphPad Prism 9.0 (GraphPad Software, Boston, MA, USA) was used for statistical analyses. All results are shown as mean ± standard deviation (SD). The number of samples (n) represents the number of independent biological replicates. All RT-qPCR and ELISA assays were performed with technical triplicates (repeated measurements of the same sample) to ensure reproducibility. Pairwise comparisons between two groups were analyzed via Student's

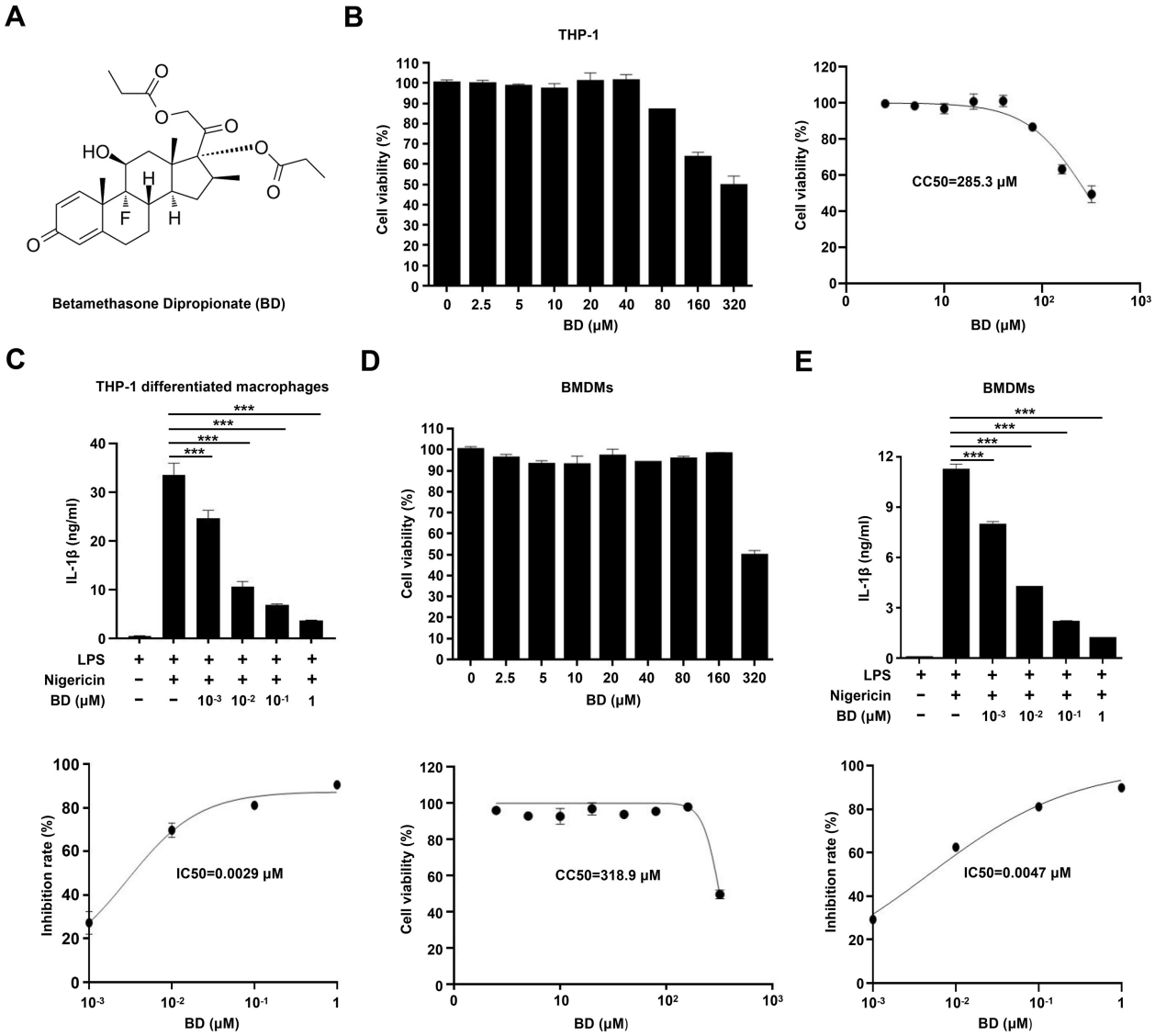

Fig. 1. BD effectively inhibits NLRP3 inflammasome activation. (A) BD's molecular structure. (B) THP-1 cells were pretreated with BD (2.5–320 μM, 24 h), followed by evaluation of cytotoxicity using a CCK-8 assay kit. (C) After pretreatment with BD (10^{-3}–1 μM, 12 h), cells were treated with LPS, followed by exposure to nigericin. Secreted IL-1β levels were determined via ELISA. (D) BMDMs were pretreated with BD (2.5–320 μM, 24 h), followed by evaluation of cytotoxicity using a CCK-8 assay kit. (E) After pretreatment with BD (10^{-3}–1 μM, 1 h), cells were treated with LPS and nigericin, after which IL-1β levels were measured. ***$p < 0.001$.

t-test, whereas one-way ANOVAs were applied for comparisons involving multiple groups, and two-way ANOVAs were adopted when the grouping system involved two factors.

3. Results

3.1 BD Effectively Inhibits NLRP3 Inflammasome Activation

BD is a glucocorticoid steroid with anti-inflammatory properties that has been employed to treat multiple inflammatory skin diseases in clinical settings [32] (Fig. 1A). The cytotoxicity of BD was initially assessed in THP-1 cells, yielding a 50% cytotoxic concentration (CC_{50}) of 285.3 μM (Fig. 1B). ELISA indicated that BD dose-dependently decreased secreted IL-1β levels in the culture supernatant of THP-1 differentiated macrophages, with a half-maximal inhibitory concentration (IC_{50}) of 0.0029 μM (Fig. 1C). Similar analyses were performed in BMDMs, yielding a CC_{50} of 318.9 μM (Fig. 1D) and an IC_{50} of 0.0047 μM for BD (Fig. 1E). Collectively, these findings indicated that in macrophages, BD effectively suppresses NLRP3 inflammasome activation.

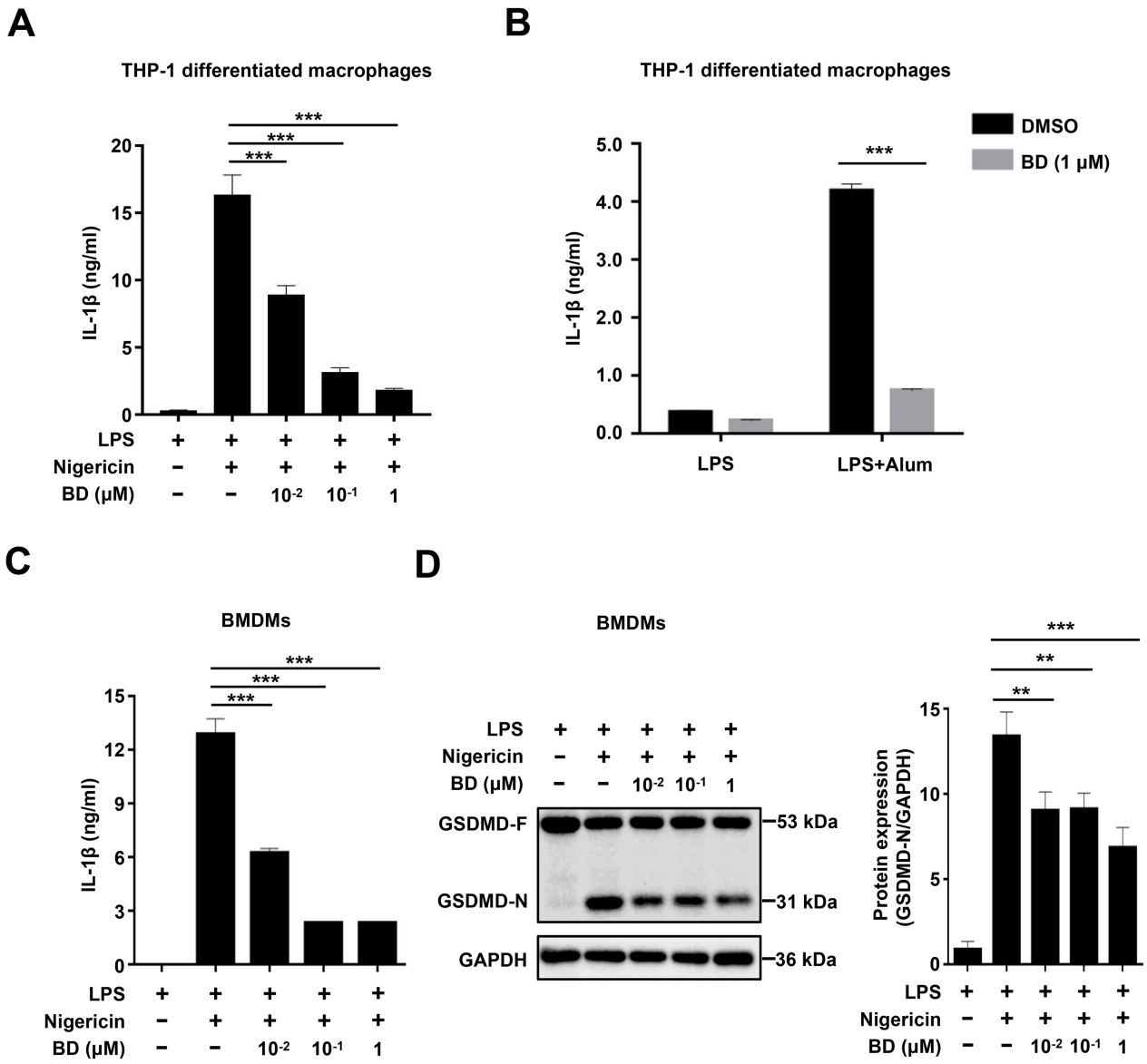

Fig. 2. BD inhibits IL-1β secretion and GSDMD-mediated pyroptosis. (A–C) Cells were pretreated with BD prior to stimulation with LPS and nigericin or alum. and the IL-1β concentration in the culture supernatants was determined via ELISA. (D) Western blotting analysis of GSDMD-FL and GSDMD-N protein levels in BMDMs, with corresponding quantification. $^{**}p < 0.01$; $^{***}p < 0.001$.

3.2 BD Inhibits IL-1β Secretion and GSDMD-Mediated Pyroptosis

BD pretreatment was conducted on THP-1 differentiated macrophages and BMDMs, followed by stimulation using LPS and nigericin to assess the inhibitory impact of BD on NLRP3 inflammasome activation. ELISA results demonstrated that BD dose-dependently suppressed mature IL-1β secretion in both cell types (Fig. 2A,C). We also found that BD significantly inhibited secreted IL-1β induced by additional NLRP3 agonists, including aluminum (Alum), in THP-1 differentiated macrophages (Fig. 2B). Moreover, BD dose-dependently suppressed the cleavage of GSDMD triggered by nigericin, thereby reducing the levels of GSDMD-N (N-terminal fragment of GSDMD) in BMDMs (Fig. 2D). These results demonstrate that BD inhibits IL-1β secretion and GSDMD-mediated pyroptosis.

3.3 BD Suppresses NLRP3 Inflammasome Activation by Reducing pro-IL-1β Expression

We examined the mechanism by which BD suppresses NLRP3 inflammasome activation in both THP-1 differentiated macrophages and BMDMs. RT-qPCR analysis revealed that BD dose-dependently decreased *pro-IL-1β* mRNA level in both types of cells (Fig. 3A,B). Consistently, BD dose-dependently decreased pro-IL-1β protein level but did not markedly downregulate NLRP3 or Casp-1

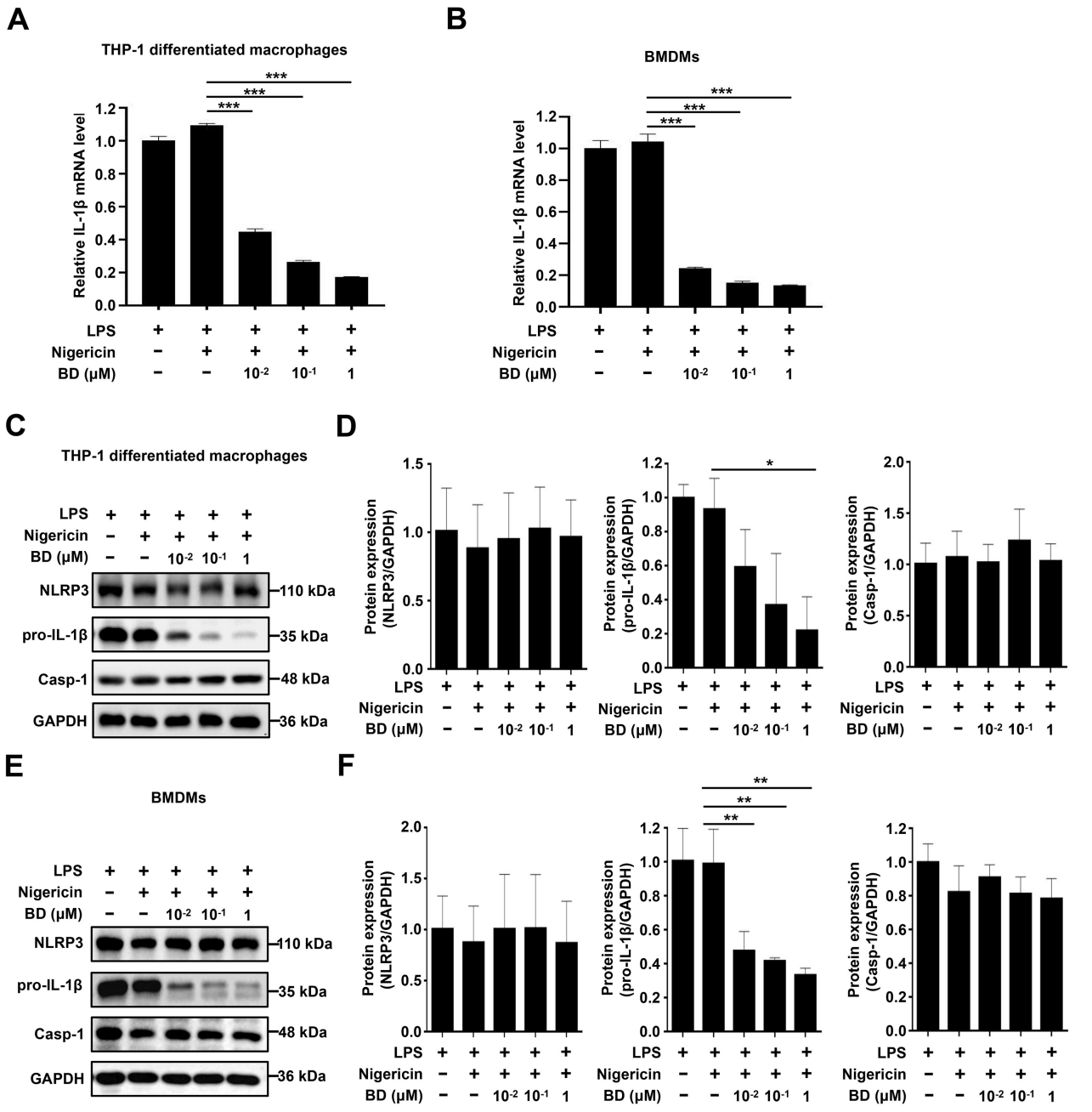

Fig. 3. BD suppresses NLRP3 inflammasome activation by reducing pro-IL-1β expression. (A,B) The level of *pro-IL-1β* mRNA was quantified by RT-qPCR. (C,D) WB analysis of NLRP3/pro-IL-1β/Casp-1 levels in THP-1 differentiated macrophages with corresponding quantification. (E,F) WB analysis of NLRP3/pro-IL-1β/Casp-1 levels in BMDMs with corresponding quantification. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

protein expression (Fig. 3C,D). These experiments were repeated in BMDMs, yielding consistent results (Fig. 3E,F). All results suggest that BD is capable of attenuating pro-IL-1β expression, thus inhibiting NLRP3 inflammasome activation.

3.4 BD Reduces pro-IL-1β Expression by Inhibiting NF-κB Signaling Pathway

In BMDMs, BD was found to suppress ATP-triggered NLRP3 inflammasome, poly(dA:dT)-induced AIM2 inflammasome, and flagellin-stimulated NLRC4 inflammasome activation (Fig. 4A). NLRP3 inflammasome activation depends on the priming signal that promotes pro-IL-1β expression, and AIM2/NLRC4 inflammasome activa-

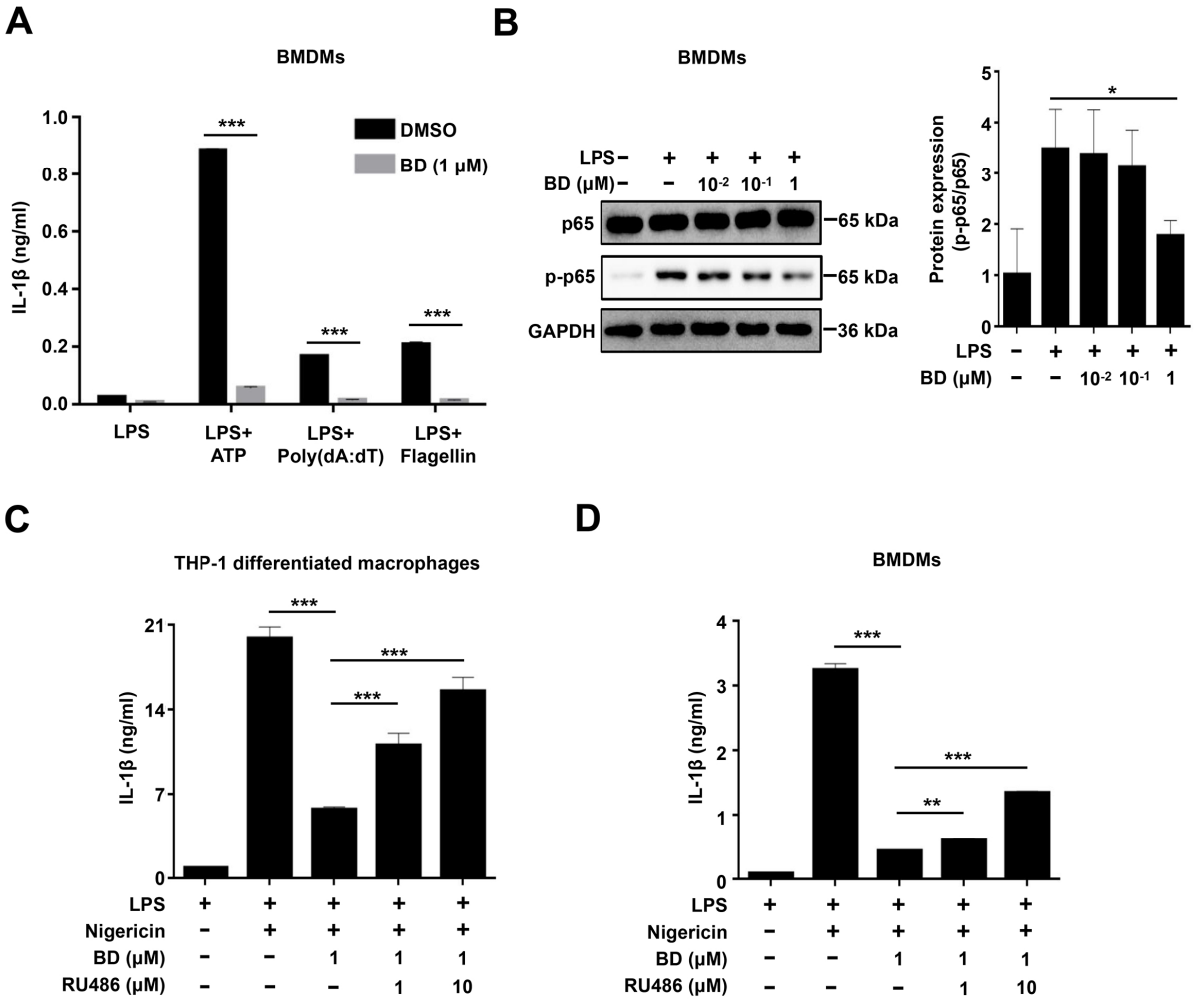

Fig. 4. BD reduces pro-IL-1β expression by inhibiting NF-κB signaling pathway. (A) BMDMs were incubated with BD, and then treated with LPS and ATP/poly(dA:dT)/flagellin. The concentration of IL-1β was determined via ELISA. (B) BMDMs were pretreated with BD and then stimulated with LPS. Then, p-p65 and p65 protein levels were detected and quantified by WB. (C,D) RU486 was administered 15 min before BD supplementation in both cell types, and IL-1β levels were analyzed by ELISA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

tion also depends on such a signal. The NF-κB signaling cascade is pivotal in regulating pro-IL-1β expression [36]. The phosphorylated p65 (p-p65) protein serves as a marker of NF-κB signaling pathway activation [37]. BD treatment lowered p-p65 expression triggered by LPS without affecting the level of total p65 protein (Fig. 4B). RU486, a well-established antagonist of the glucocorticoid receptor (GR), was applied as a pretreatment prior to BD administration, revealing that BD-mediated IL-1β inhibition was reduced as the concentration of RU486 increased in both THP-1 differentiated macrophages and BMDMs (Fig. 4C,D). These results indicated that BD may still suppress inflammasome activation, but this suppression is not fully GR-dependent. Overall, the results suggest that BD reduces pro-IL-1β expression by inhibiting NF-κB signaling pathway.

3.5 BD may not Directly Affect the Assembly of NLRP3 Inflammasome

We investigated whether BD directly regulates NLRP3 inflammasome assembly, a preliminary analysis of the ability of BD to inhibit interactions between the main components of NLRP3 inflammasome was determined by Co-IP. Co-IP results indicated that BD did not disrupt the NLRP3-NEK7/NLRP3-ASC/NLRP3-NLRP3 interactions (Fig. 5A–C). We subsequently performed a DARTS assay to investigate the binding affinity of BD for the main components of NLRP3 inflammasome. No detectable protection was observed under these DARTS conditions in the HEK293T cells overexpressing NLRP3/ASC/caspase-1 (Fig. 5D–F). Overall, these results suggested that BD may not directly interfere with NLRP3 inflammasome assembly during the activation signal.

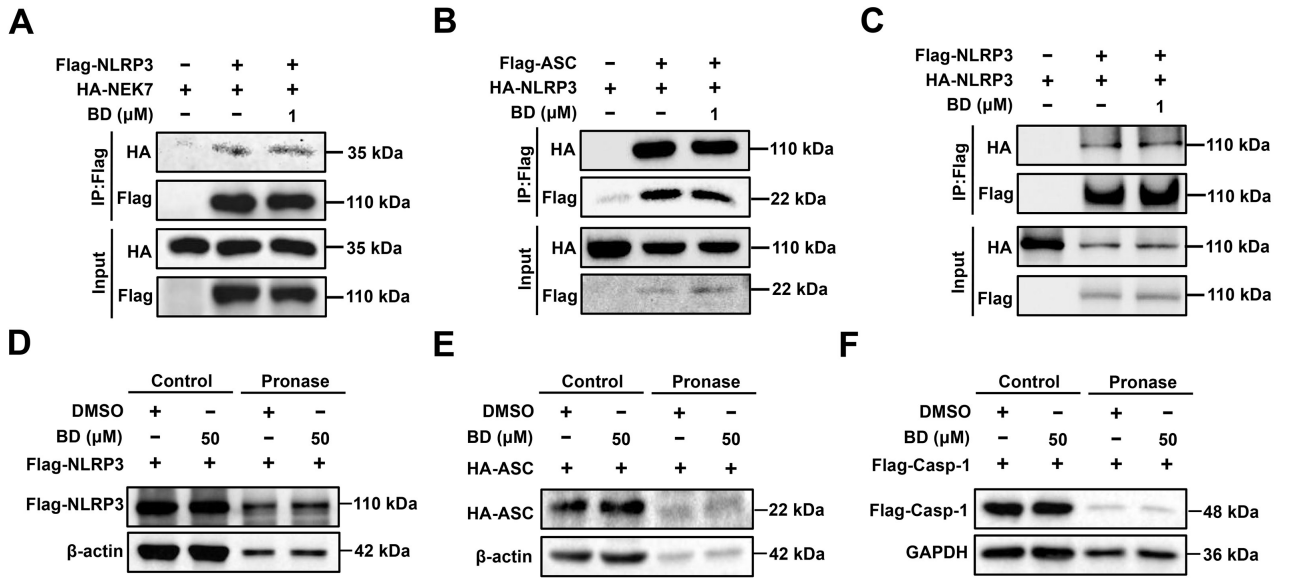

Fig. 5. BD may not directly affect the assembly of NLRP3 inflammasome. (A–C) The specified plasmid constructs were introduced into HEK293T cells, and the binding association between NLRP3-NEK7 (A), NLRP3-ASC (B), or NLRP3-NLRP3 (C) and BD (1 μM) were determined by Co-IP. (D–F) The cell lysates were then treated with BD (50 μM) and pronase. Overexpressed NLRP3 (D), ASC (E), and Casp-1 (F) were analyzed by DARTS.

3.6 BD Alleviates LPS-induced Systemic Inflammation in Mice

LPS-induced systemic inflammation is a well-established model of NLRP3 inflammasome-associated inflammatory diseases [38,39,40]. To evaluate anti-inflammatory effect of BD in mice, the mice were pretreated with BD and subsequently injected with LPS (Fig. 6A). ELISA results indicated that BD effectively reduced the concentrations of IL-1β and TNF-α in the serum (Fig. 6B). Using a similar model with lower-dose LPS treatment (Fig. 6C), we monitored the survival rate and body weight changes of the mice. We found that BD increased survival rate of the mice (Fig. 6D). For evaluating the therapeutic effect of BD in a relevant setting, the mice were treated with LPS and then given an injection of BD (Fig. 6E). We also found that BD significantly increased survival rate of the mice over 96 h (Fig. 6F). Overall, these results demonstrated that BD effectively alleviated LPS-induced systemic inflammation.

4. Discussion

Through this research, we observed that BD significantly reduced pro-IL-1β expression, mature IL-1β secretion, and GSDMD-mediated pyroptosis. Additionally, during the priming signal, it markedly inhibited pro-IL-1β expression by downregulating NF-κB signaling pathway, while it did not affect NLRP3 levels. Co-IP and DARTS assays indicated that BD likely has no direct effect on the assembly of NLRP3 inflammasome during the activation signal. Strikingly, BD administration *in vivo* effectively decreased the concentrations of IL-1β and TNF-α in the serum and markedly improved survival rate of treated mice.

Glucocorticoids that are routinely used in the clinic, such as dexamethasone, exert broad anti-inflammatory effects by activating the GR and modulating NF-κB signaling activity [41,42]. However, dexamethasone-induced Dectin-1 activation has been shown to enhance NLRP3 inflammasome activation and GSDMD-mediated pyroptosis [43]. Unlike dexamethasone, we found that BD exerts its effect by suppressing pro-IL-1β expression, thereby inhibiting NLRP3 inflammasome activation and GSDMD-mediated pyroptosis. These observations imply that distinct glucocorticoids may regulate NLRP3 inflammasome activation via divergent signaling pathways. However, the precise mechanisms by which BD suppresses NLRP3 inflammasome activation need to be further investigated.

Activation of NF-κB signaling pathway is a critical step in the priming of NLRP3 inflammasome activation, as it promotes the transcriptional upregulation of pro-IL-1β and NLRP3, thereby providing the necessary molecular basis for inflammasome assembly [36,44,45]. Interestingly, BD could suppress pro-IL-1β expression while exerting no influence on NLRP3 levels. The precise mechanisms by which BD specifically inhibits pro-IL-1β expression need to be further investigated. By detecting secreted mature IL-1β levels, we also observed that BD inhibits AIM2/NLRC4 inflammasome activation. These results suggest that BD may suppress pro-IL-1β expression in the priming signal of NLRP3/AIM2/NLRC4 inflammasomes. However, this hypothesis requires further validation and investigation.

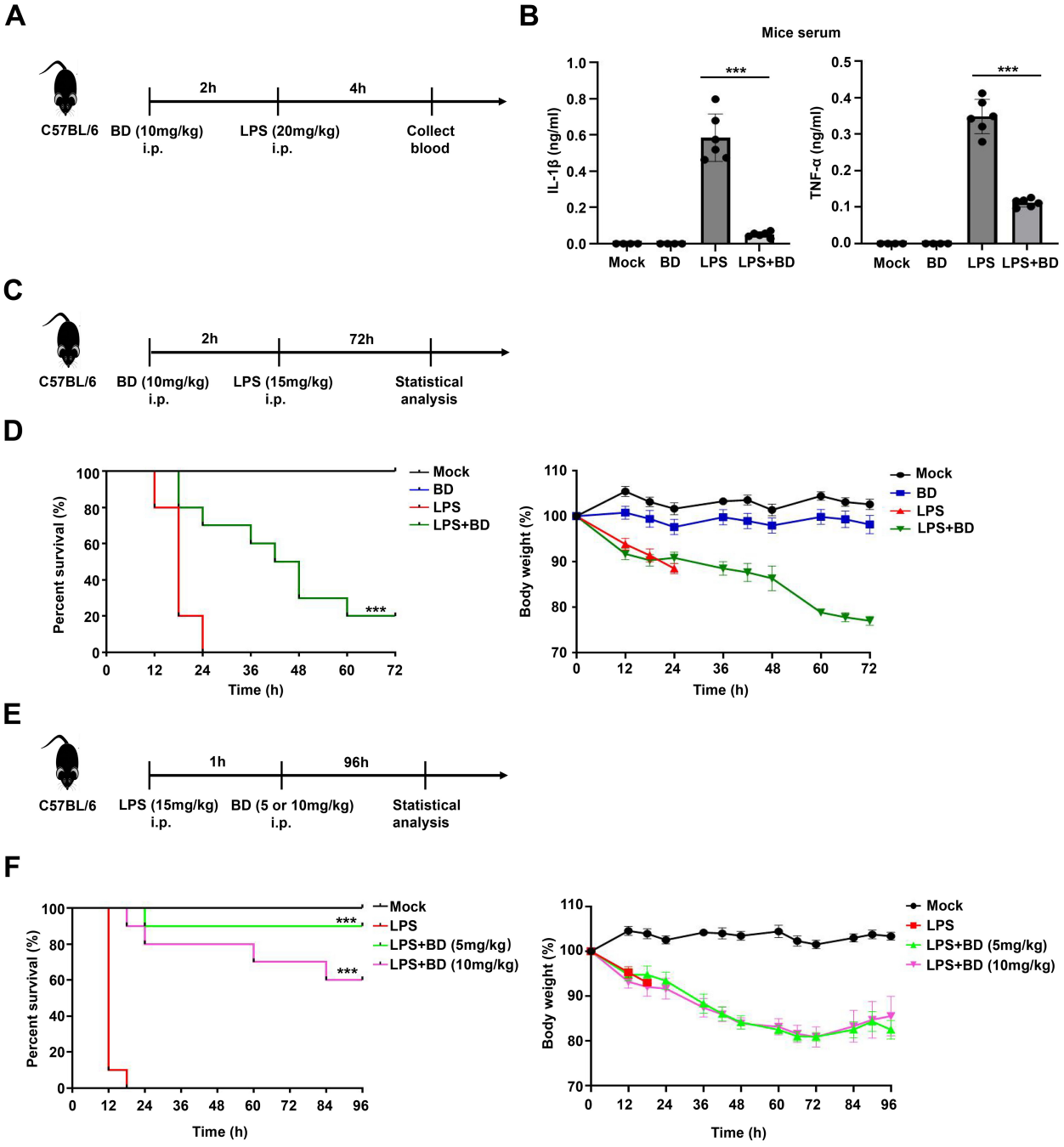

Fig. 6. BD alleviates LPS-induced systemic inflammation in mice. (A,B) BD was administered to female mice 2 h before LPS challenge (n = 4 for Mock/BD group; n = 6 for LPS/LPS+BD group). Mouse serum was then isolated, and the concentrations of IL-1β and TNF-α were quantified by ELISA. (C,D) Following pretreatment with BD, female mice were injected with LPS (n = 6 for Mock/BD group; n = 10 for LPS/LPS+BD group). Survival and body weight were monitored every 6–12 h for 72 h. (E,F) Female mice were treated with LPS, followed by BD treatment (n = 6 for Mock group; n = 10 for LPS/LPS+BD (5 mg/kg)/LPS+BD (10 mg/kg) group). Survival rate and body weight were monitored at 6–12 h intervals for 96 h. Data are presented as mean ± SD. ELISA results were compared using one-way ANOVAs, while survival rates were analyzed using the log-rank (Mantel-Cox) test. ***$p < 0.001$.

It has been reported that GR activation exerts anti-inflammatory effects by downregulating various inflammatory cytokines, and this regulation is mediated via blockade of NF-κB signaling pathway [46]. To test whether BD exerts its effects in a GR-dependent manner, we used the GR-specific antagonist RU486. The results indicated that BD still retained a partial inhibitory effect on the activation of NLRP3 inflammasome independent of GR, and that the GR

pathway only mediated a portion of the anti-inflammatory effects of BD. Therefore, further investigation should focus on the impact of BD on other signaling proteins in the priming signal, as such efforts will help elucidate how BD precisely regulates NLRP3 inflammasome activation.

To preliminarily explore whether BD affects NLRP3 inflammasome assembly, we performed Co-IP and DARTS assays using HEK293T cells overexpressing NLRP3/ASC/caspase-1/NEK7. These findings suggested that BD does not seem to directly disrupt the assembly of NLRP3 inflammasome. However, since we did not explore the effect of BD on the assembly of endogenous NLRP3 inflammasomes in macrophages, the possibility that BD interacts upstream of NLRP3 inflammasome assembly cannot be excluded. This suggests that BD may also affect NLRP3 inflammasome assembly via regulating upstream signals, including reactive oxygen species (ROS) production, Ca^{2+} influx and K^{+} efflux [47,48]. The precise molecular basis remains to be clarified.

Our study shows that BD inhibits pro-IL-1β expression during the priming signal, thereby suppressing mature IL-1β secretion. Reduced mature IL-1β levels attenuate the positive feedback loop that sustains NLRP3 inflammasome activity, thus inhibiting NLRP3 inflammasome activation [49,50]. Consequently, by inhibiting the priming signal, BD reduces downstream mature IL-1β levels, which in turn indirectly suppresses NLRP3 inflammasome assembly through attenuation of this positive feedback mechanism. However, this hypothesis requires further validation and investigation.

The LPS-induced systemic inflammation model is a classic approach that is widely used to study acute inflammation. However, it is important to note that it is not specific for NLRP3, as LPS also induces other inflammatory mediators like TNF-α. Our study indicated that BD reduced the serum concentrations of IL-1β and TNF-α in the LPS-induced systemic inflammation. Therefore, in this model, it is insufficient to demonstrate that BD restrains NLRP3 inflammasome activation. Aside from the acute inflammation model, many chronic inflammation models (such as type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD)) are also NLRP3 inflammasome-associated inflammatory disease models [51,52]. Considering its limitations in simulating chronic pathology, we will adopt these chronic inflammation models in our future investigations to probe the actions of BD on NLRP3 inflammasome activation. Meanwhile, future studies should adopt NLRP3-knockout mice models to further verify BD's modulatory role in NLRP3 inflammasome activation.

BD is a glucocorticoid that is clinically indicated primarily for treating psoriasis. It exerts its therapeutic efficacy by targeting pro-inflammatory cytokines and facilitating keratinocyte differentiation, as well as by inhibiting IL-23/IL-17 pathogenic T cell axis and inducing regulatory $CD8^{+}$ T cells [53,54,55,56]. Our results demonstrate that BD suppresses NLRP3 inflammasome activation and GSDMD-mediated pyroptosis in macrophages. However, we have only verified its effects in THP-1 cells and BMDMs. Therefore, subsequent studies should examine its effects in human primary macrophages and other immune cells to validate these observations. Moreover, in addition to GR, the specific protein target through which BD suppresses NLRP3 inflammasome activation remains to be identified. At the animal level, BD has been shown to ameliorate LPS-induced systemic inflammation in mice. Future studies are needed to examine its effects in more inflammatory disease models. In summary, BD can serve as a potential therapeutic candidate for inflammatory diseases. Nevertheless, there is still a long way to go before BD can be used clinically to treat the inflammatory diseases.

5. Limitations

Several shortcomings of the present work should be pointed out. First, we found that BD significantly reduces pro-IL-1β expression by suppressing NF-κB signaling pathway. However, potential contributions of other signaling pathways, such as activator protein-1 (AP-1) signaling pathway, to this effect remain to be established. Further comparative analyses of multiple signaling pathways are thus warranted to clarify the dominant role of NF-κB signaling pathway. In addition, p50 and p65 phosphorylation status should be examined in multiple cell models, including THP-1-differentiated macrophages and BMDMs. Second, our conclusion that BD may not directly affect NLRP3 inflammasome assembly is based on limited Co-IP and DARTS assays from HEK293T cells overexpressing the relevant proteins, which may not fully recapitulate physiological assembly. Moreover, whether BD modulates upstream triggers such as K^{+} efflux or ROS production remains unclear. Thus, we cannot exclude the possibility that BD may also inhibit the activation signal for NLRP3 inflammasome.

6. Conclusion

In conclusion, this study provides the first evidence that BD suppresses NLRP3 inflammasome activation, mature IL-1β secretion, and GSDMD-mediated pyroptosis. Moreover, we found that BD was able to significantly reduce pro-IL-1β expression via suppressing NF-κB signaling pathway, whereas it had no direct effect on the assembly of NLRP3 inflammasome, thus modulating the priming signal rather than the activation signal for NLRP3 inflammasome activation. We also determined that BD mitigates LPS-induced systemic inflammation, reducing inflammatory cytokines (such as IL-1β and TNF-α) and elevating survival rate of treated mice. Collectively, these results suggest that BD may represent an effective therapeutic candidate for NLRP3 inflammasome-associated inflammatory diseases.

Availability of Data and Materials

All raw experimental data and research materials related to this study are available from the corresponding author upon reasonable request.

Author Contributions

WH: Writing-original draft, Methodology, Investigation, Formal analysis. SX: Writing-original draft, Methodology, Investigation, Data curation. XR: Investigation, Methodology. YL: Investigation, Methodology. WL: Formal analysis, Methodology. QP: Methodology, Resources. SH: Resources, Conceptualization, Data curation, Writing-review & editing. BS: Conceptualization, Supervision, Resources, Writing-review & editing. PW: Conceptualization, Funding acquisition, Writing-original draft, Writing-review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The experiments were conducted at the National Research Council of Guide for the Care and Use of Laboratory Animals. The study was carried out in accordance with the ARRIVE guidelines. All animal experimental procedures were performed in strict accordance with the protocols approved by the Animal Ethics Committee of Jianghan University and relevant institutional guidelines. (Approval Number: JHDXKJLL2025-176).

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL51536.

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