# Inhibition of granzyme B activity blocks inflammation induced by lipopolysaccharide through regulation of endoplasmic reticulum stress signaling in NK92 cells

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**Abstract.** Granzyme B (GrB) is a serine protease that is expressed in the lytic granules of natural killer (NK) cells and cytotoxic T lymphocytes (CTL), and which has been widely reported to serve a crucial role for target cell apoptosis. GrB may serve a non-cytotoxic role in inflammation, but the evidence remains unclear. The present study aimed to establish an inflammatory cell model by using NK92 cells stimulated with lipopolysaccharide (LPS) to investigate whether GrB was involved in the development of inflammation. The extracellular levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and GrB were examined by ELISA, and it was demonstrated that LPS treatment increased the extracellular levels of TNF-α, IL-1β and GrB, and these increased expression levels were inhibited by pretreatment with the GrB inhibitor serpin A3N (SA3N). The protein expression levels of glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), nuclear factor-κB (NF-κB), inhibitor of NF-κB (IκBα) and GrB were examined by western blot analysis. The results demonstrated that LPS stimulation increased the expression levels of GRP78, CHOP, NF-κB and GrB, and decreased the expression of IκBα, and these changes were inhibited by SA3N, which indicated that inhibition of GrB activity may suppress endoplasmic reticulum (ER) stress signaling. Therefore, it was suggested that GrB may be a potential pro-inflammatory factor, and inhibition of GrB activity may aid the prevention of the development of inflammation by suppressing ER stress signaling.

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## Introduction

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) provide crucial defense against virus-infected cells and tumor cells; NK cells and CTLs target tumor cells and induce apoptosis (1). Granzyme B (GrB) belongs to a family of serine proteases that are expressed in the granules of activated CTLs and NK cells that induce apoptosis (2,3). GrB is also expressed in non-lymphoid lineage cells, including chondrocytes, neutrophils, keratinocytes and macrophages (4). It has been reported that the intracellular serpin proteinase inhibitor 9 may protect monocytes and macrophages from misdirected GrB in the lipopolysaccharide (LPS)-induced inflammatory process (5). GrB is considered a 'natural born killer' that functions at multiple points, including directing proteolytic processing and activation of procaspase-3 and -7, to initiate the death of the harmful target cells, including allogeneic, virus-infected and tumor cells (6). GrB directly targets caspase-3 and initiates the caspase cascade, leading to DNA fragmentation and apoptosis. Alternately, in the absence of caspase activity, GrB may still be able to initiate mitochondrial events through the cleavage of the B-cell lymphoma 2 homology domain 3-interacting domain death agonist protein (7), which is important to cell death and is susceptible to proteolytic cleavage by GrB as well as caspases, calpains and cathepsins (8). A recent report suggested that GrB may also possess non-cytotoxic roles under inflammatory circumstances as well as possible functions in the extracellular space (9). In addition, previous studies have reported that GrA and GrB may influence the production of pro-inflammatory cytokines; however, the molecular targets of these proteases in inflammation remain unknown (10,11). Whether GrB is involved in and promotes the development of inflammation also remains to be elucidated.

LPS is a component of the Gram-negative bacteria cell wall that is known to induce inflammation in a number of physiological and experimental settings. A previous study reported that inhibition of endoplasmic reticulum (ER) stress

alleviated LPS-induced lung inflammation through modulation of the nuclear factor-κB (NF-κB) signaling pathway (12). Serine protease inhibitor A3N (serpin A3N; SA3N) is an extracellular inhibitor of GrB that has been demonstrated to possess multiple biological functions, including the attenuation of muscular dystrophy in mice (13), neuropathic pain (14) and GrB-mediated decorin cleavage and rupture (15); it also induces neuroprotection *in vitro* and *in vivo* (16).

The present study established an inflammatory cell model by using NK92 cells stimulated with LPS, and demonstrated that the LPS-induced inflammatory response was prevented by the inhibition of GrB activity.

## Materials and methods

*Chemicals*. LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); α-minimum essential medium (MEM) and antibodies against glucose-regulated protein 78 (GRP78; cat. no. sc-376768), C/EBP homologous protein (CHOP; cat. no. sc-4066) and β-actin (cat. no. sc-8432) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against GrB (cat. no. 4275S), NF-κB (cat. no. 8242S) and inhibitor of NF-κB (IκBα; cat. no. 9242S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

*Cell culture*. NK92 cells were supplied from Kunming Institute of Zoology (Kunming, China; the human NK92 cell were established from a donor who suffered from malignant non-Hodgkin's lymphoma and who gave written informed consent). NK92 cells were maintained in α-MEM medium with 5% heat-inactivated fetal bovine serum (FBS) and 10% heat-inactivated fetal horse serum (both Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin) at 37˚C in a humidified 5% $CO_2$ atmosphere.

*LPS-induced inflammation*

*Concentration dependence of LPS*. NK92 cells in logarithmic growth phase were incubated in 6-well culture plates and divided into 6 groups, each treated with 1, 10, 50, 100, and 500 ng/ml LPS, along with an untreated control group. Following medium exchange, 100 $\mu$l of the different concentrations of LPS were added to the 6 different LPS treatment groups, and 100 $\mu$l α-MEM medium containing 5% heat-inactivated FBS, 10% heat-inactivated fetal horse serum and antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin) were added to the control group. The cells were then incubated at 37˚C in a humidified 5% $CO_2$ atmosphere for 24 h. The cell culture medium was collected and precipitated by centrifugation for 5 min (750 x g, 4˚C) and the clear supernatant extract was analyzed using ELISA. Experiments were performed in triplicate.

*Time dependence of LPS*. According to the optimal concentration determined by the previous LPS concentration dependence test, 100 ng/ml LPS was added to every test group. NK92 cells in logarithmic growth phase were incubated in 6-well culture plates and divided into 5 groups, which were incubated with LPS for 0 (control), 12, 24, 48 or 72 h. The cell culture medium was collected and precipitated by centrifugation for 5 min (750 x g, 4˚C) and the clear supernatant extract analyzed using ELISA. Experiments were performed in triplicate.

*Role of SA3N on LPS-induced inflammation*. NK92 cells in logarithmic growth phase were incubated in 6-well culture plates and divided into 4 groups: control, LPS, LPS + SA3N (GeneCopoeia, Inc., Rockville, MD, USA) and SA3N groups. The cells were treated with SA3N (20 $\mu$M) for 30 min at 37˚C followed by stimulation with 100 ng/ml LPS for 24 h at 37˚C. Cell culture medium was collected and precipitated by centrifugation for 5 min (750 x g, 4˚C) the clear supernatant extract was analyzed using ELISA, and the sedimented cells were analyzed by western blotting. Experiments were performed in triplicate.

*ELISA analysis*. The levels of TNF-α, IL-β and GrB in the clear supernatant extract were quantified using Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA; TNF-α, cat. no. DTA00C; IL-β, cat. no. DLB50; GrB, cat. no. DY2906-05), according to the manufacturer's protocols. Plates were read in an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm. The values obtained were plotted onto the standard plot prepared by using serial dilutions of the standard provided with the kit and TNF-α, IL-β and GrB concentrations were calculated.

*Western blot analysis*. Cells were plated in 6-well plates at a density of $1x10^5$ cells/well. Total protein was extracted using lysis buffer and incubated on ice for 1 h. Protein lysates were prepared using a solubilizing solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM ethylene-bis(oxyethyl enenitrilo)-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM β-glycerol phosphate, and 1 mg/ml leupeptin]. Protein concentrations were determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). Samples were separated on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was rinsed with PBS and nonspecific sites were blocked by incubating the membrane with blocking buffer (PBS + 0.1% Tween-20, containing 10% nonfat milk) overnight at 4˚C or for 2 h at room temperature. Membranes were subsequently incubated with primary antibodies to GrB (1:1,500), GRP78 (1:1,000), CHOP (1:1,500), NF-κB (1:1,000), IκBα (1:1,500), and β-actin (1:1,500) for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated anti-mouse (1:10,000; cat. no. MA5-16308) or anti-rabbit (1:10,000; cat. no. 42-6600) immunoglobulin G (KPL, Inc., Gaithersburg, MD, USA) for 1 h at room temperature. Protein bands were visualized using an ECL Western Blot Detection kit (EMD Millipore). ImageJ V1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used for the densitometric analysis. All protein bands are normalized to β-actin. Three independent experiments were performed.

*Statistical analysis*. Data were analyzed by using SPSS software 16.0 (SPSS, Inc., Chicago, IL, USA) and were expressed as the mean ± standard error of the mean. One-way analysis of

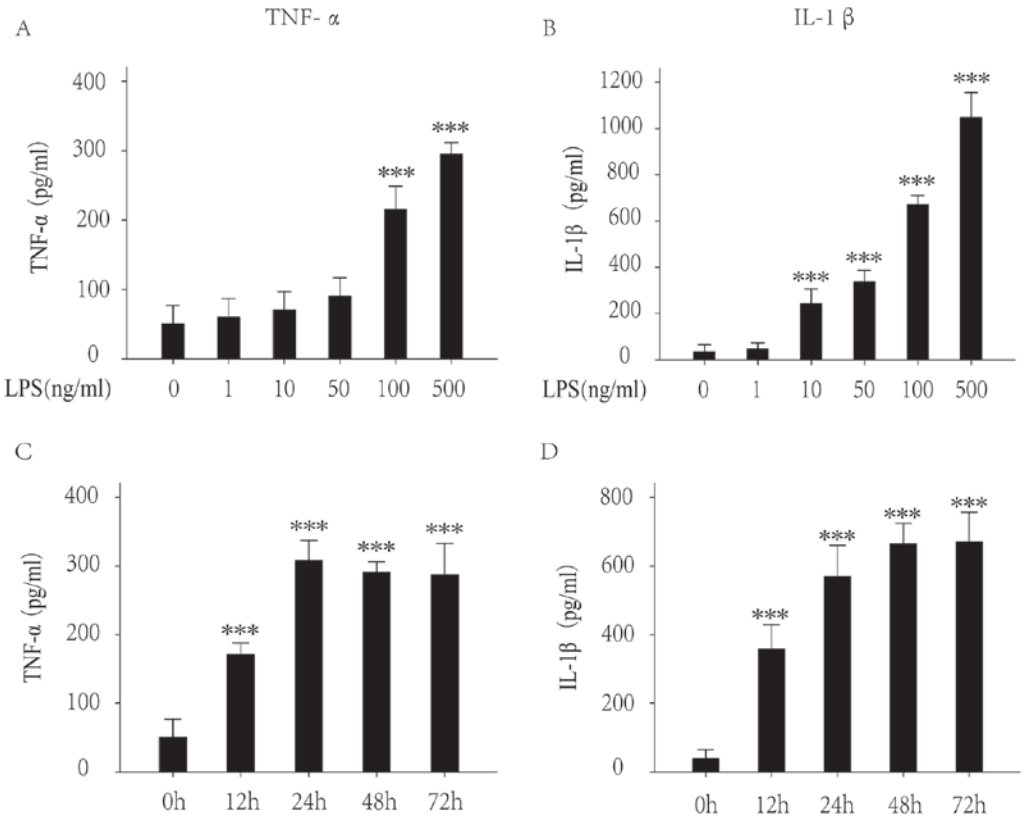


Figure 1. LPS-induced inflammatory response in NK92 cells. (A) LPS treatment significantly increased the extracellular levels of TNF-α at 100 and 500 ng/ml following incubation for 24 h. (B) LPS significantly increased the extracellular levels of IL-1β at 10, 50, 100 and 500 ng/ml following incubation for 24 h. (C and D) LPS significantly increased the extracellular levels of (C) TNF-α and (D) IL-β at 100 ng/ml following incubation for 12, 24, 48 and 72 h. ***P<0.001 vs. 0 ng/ml (and 0 h) untreated control cells. IL-1β, interleukin-1β; LPS, lipopolysaccharide; NK, natural killer; TNF-α, tumor necrosis factor-α.

variance followed by a Bonferroni post hoc multiple comparison test was used to compare control and treated groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*LPS-induced inflammatory response in NK92 cells*. TNF-α and IL-1β are key cytokines involved in inflammation. The extracellular levels of TNF-α and IL-1β were investigated by ELISA following LPS treatment (1, 10, 50, 100 and 500 ng/ml) for 24 h. The results demonstrated that 100 ng/mg LPS significantly increased the levels of TNF-α (Fig. 1A) and IL-1β (Fig. 1B). The extracellular levels of TNF-α and IL-1β following LPS treatment (100 ng/ml) for various times (0, 12, 24, 48 and 72 h) were subsequently investigated. The results demonstrated that LPS significantly increased the levels of TNF-α (Fig. 1C) and IL-1β (Fig. 1D) following treatment for 12 h, and the levels become stable at 24 h. Thus, 100 ng/ml for 24 h was set as the best condition for the following experiments.

*SA3N pretreatment prevents the expression and exocytosis of GrB by LPS*. The expression of GrB was examined by western blotting and the results demonstrated that LPS increased the expression of GrB, whereas SA3N (20 $\mu$M) pretreatment suppressed the increase by LPS (Fig. 2A). In Fig. 2A, the bands that appear above GrB may be a larger fragment of GrB or may also be a nonspecific binding, as the active GrB is derived from the precursor GrB by deglycosylation. Generally, the molecular weight of the precursor GrB is 35 kDa, and the active GrB is 32 kDa. The extracellular levels of GrB were examined by ELISA and the results demonstrated that LPS increased the extracellular levels of GrB, whereas SA3N pretreatment suppressed the increase by LPS stimulation (Fig. 2B).

*SA3N pretreatment prevents the exocytosis of TNF-α and IL-1β induced by LPS*. The extracellular levels of TNF-α and IL-1β were examined by ELISA. The results demonstrated that LPS increased the extracellular levels of TNF-α (Fig. 3A) and IL-1β (Fig. 3B), whereas SA3N pretreatment suppressed the increases by LPS treatment. The results indicated that reduction of GrB activity may contribute to alleviate the LPS-induced inflammatory response.

*SA3N pretreatment prevents the LPS-induced changes in expression levels of GRP78, CHOP, NF-κB and IκBα proteins*. GRP78, CHOP, NF-κB and IκBα were involved in LPS-induced ER stress (12,17). It has been demonstrated that inhibition of ER mediated by NF-κB pathway may alleviate LPS-induced lung inflammation (12).Thus, the NF-κB pathway was examined. The western blotting results demonstrated that LPS stimulation increased the expression levels of GRP78,

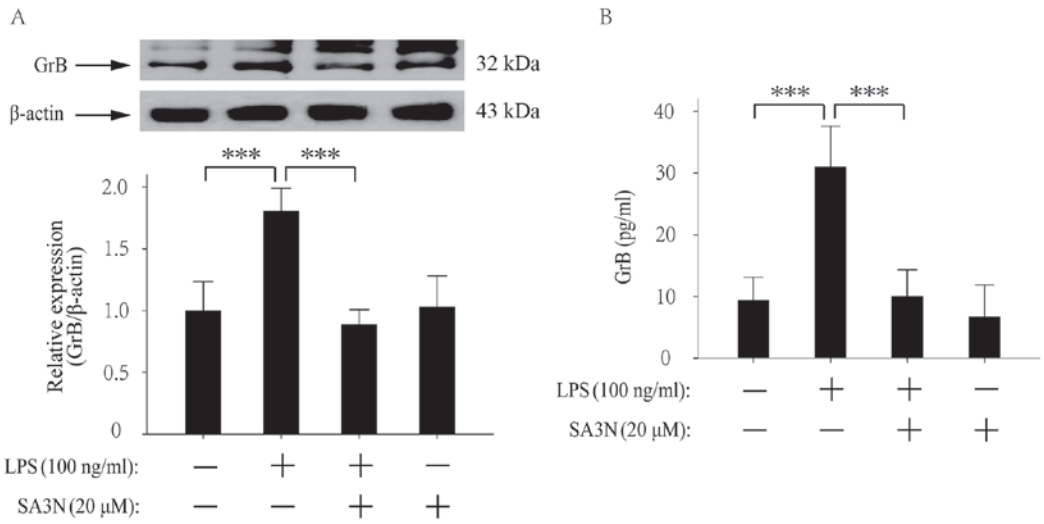


Figure 2. Role of SA3N pretreatment on the expression and exocytosis of GrB by LPS. (A) LPS treatment increased the protein expression level of GrB, whereas SA3N (20 $\mu$M) pretreatment suppressed this LPS-induced increase. (B) LPS increased the extracellular levels of GrB, and SA3N pretreatment suppressed the increase by LPS. ***P<0.001. GrB, granzyme B; LPS, lipopolysaccharide; SA3N, serpin A3N.

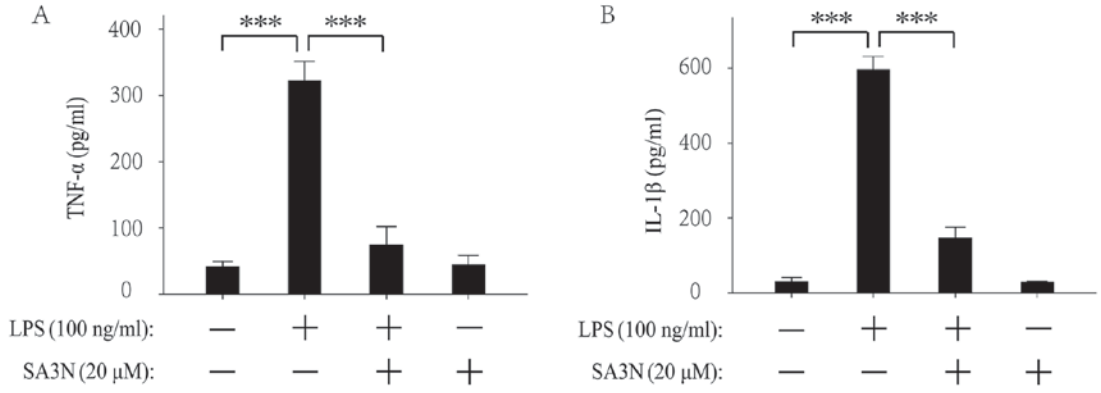


Figure 3. Role of SA3N pretreatment on the LPS-induced exocytosis of TNF-α and IL-1β. (A and B) LPS treatment increased the extracellular levels of (A) TNF-α and (B) IL-1β, whereas SA3N pretreatment suppressed these LPS-induced increases. ***P<0.001. IL-1β, interleukin-1β; LPS, lipopolysaccharide; SA3N, serpin A3N; TNF-α, tumor necrosis factor-α.

CHOP and NF-κB, and decreased the expression of IκBα, and SA3N pretreatment suppressed the changes in expression levels induced by LPS (Fig. 4A-D). The results indicated that the reduction of GrB activity alleviated LPS-induced inflammatory response may occur through the regulation of the NF-κB pathway.

## Discussion

GrB is a major constituent of CTL and NK cell granules, and the mechanism of GrB-mediated cell death has been well studied (9). Recent evidence has begun to uncover possible non-cytotoxic roles for GrB (18). Elevated levels of circulating GrB are a characteristic feature of a number of inflammatory diseases (19,20). In addition, a previous study reported that GrB may be involved in LPS-induced toxic shock, which suggested that, not only does GrB promote cell death upon delivery to target cells, but it may also function upon release into the extracellular space (21). However, it remains to be elucidated if GrB is involved in the development of inflammation. Exploring the role of GrB in the development of inflammation will provide a new strategy for the treatment of inflammatory diseases.

LPS activates innate immune cells, which leads to the production of pro-inflammatory cytokines (22). It has been reported that citrate is able to modulate LPS-induced monocyte inflammatory responses (23) and that suppression of NF-κB signaling in BV-2 microglial cells inhibits LPS-induced inflammatory responses (24). The present study demonstrated that LPS treatment significantly increased the release of inflammatory cytokines TNF-α and IL-1β, which indicated that LPS was able to induce the inflammatory response in NK92 cells. The present results also demonstrated that LPS stimulation increased the expression and release of GrB in NK92 cells, which indicated that GrB may be involved in the development of inflammation; however, SA3N pretreatment suppressed this LPS-induced increase. SA3N forms a complex and stable covalent bond with GrB that results in the inhibition of the enzymatic activity of the protease (25). In the present study, treatment with SA3N alone did not change the expression of GrB, whereas pretreatment

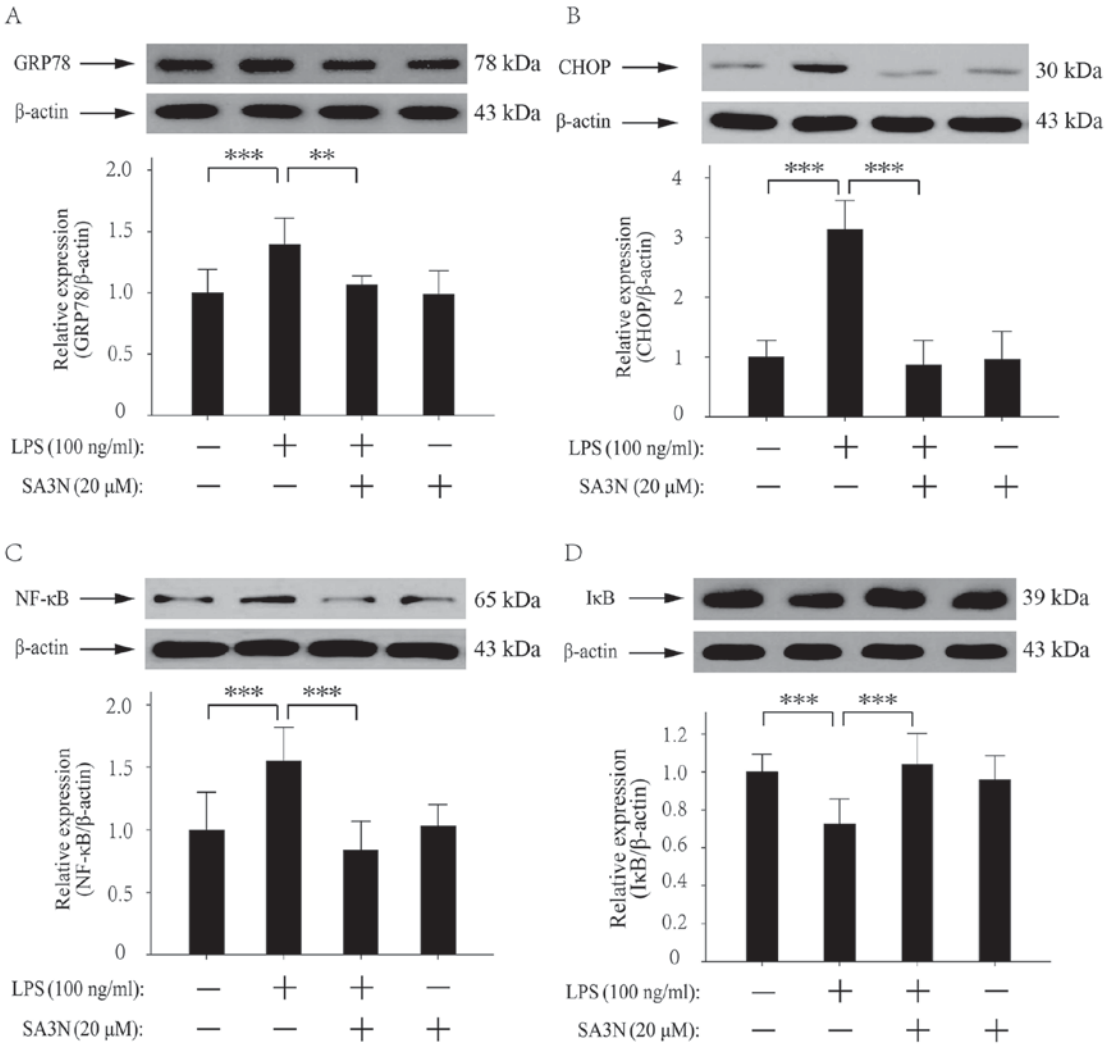


Figure 4. Role of SA3N pretreatment on the LPS-induced changes of GRP78, CHOP, NF-κB and IκBα protein expression levels. LPS increased the expression levels of (A) GRP78, (B) CHOP and (C) NF-κB, whereas SA3N pretreatment suppressed the increases induced by LPS. (D) LPS treatment decreased the expression of IκBα, and SA3N pretreatment suppressed the reduction induced by LPS. $^{**}$P<0.01 and $^{***}$P<0.001. CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; IκBα, inhibitor of NF-κB; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SA3N, serpin A3N.

with SA3N prior to LPS stimulation inhibited the expression of GrB in NK92 cells, which suggested that pretreatment with SA3N may block the LPS-induced cytotoxicity mediated by GrB by suppressing the activity of GrB; this may be the reason that the expression of GrB in LPS + SA3N treated cells was not increased compared with the control group. It was also identified that LPS treatment significantly increased the levels of TNF-α and IL-1β, which were also suppressed in cells pretreated with SA3N pretreatment. To elucidate how GrB served its role on LPS-induced inflammation, ER stress was investigated.

ER stress has been reported to be induced by LPS (26). The NF-κB signaling pathway is involved in ER stress (27), and GRP78 and CHOP are markers of ER stress and are involved in the unfolding protein reaction and in the protection mechanism during ER stress (28-30). It has been reported that the increased expression of CHOP during the development of ER stress was inhibited when ER stress was blocked (30). Consistent with these reports, the results of the present study demonstrated that LPS treatment significantly induced the expression levels of GRP78 and CHOP, which were inhibited by SA3N pretreatment. NF-κB activation regulates the expression of >100 genes involved in diverse cell processes, including cell proliferation, differentiation, apoptosis and the inflammation and immune responses (31,32). A previous study demonstrated that CHOP induced cell inflammatory responses by activating NF-κB (33). Results from the present study indicated that LPS treatment induced the expression of NF-κB and decreased the expression of IκBα, which indicating that LPS induced inflammatory response may be through initiating ER stress. However, inhibition of GrB activity suppressed the changes of NF-κB and IκBα expression levels induced by LPS. Thus, it was hypothesized that inhibition of GrB activity may have suppressed ER stress by blocking the NF-κB pathway, which may indicate that inhibition of GrB activity blocked the development of inflammation. In addition, it has been reported that TNF-α mediated the activation of NF-κB in various disease processes, including inflammation and cell apoptosis (34). In the present study, inhibition of GrB activity blocked the exocytosis of TNF-α, and it is therefore suggested that the reduction of TNF-α levels may be the key point that blocked the activation of the NF-κB pathway.

SA3N is the only known GrB activity inhibitor that is secreted extracellularly (35). It has been previously reported that SA3N attenuates GrB-mediated decorin cleavage and rupture in a mouse model of aortic aneurysm (15) and that SA3N induces neuroprotection *in vitro* and *in vivo*, which has been reported to be a potentially novel therapeutic approach for inflammation-mediated neurodegenerative diseases such as multiple sclerosis (23). However, SA3N is not a GrB specific inhibitor. It has been reported that SA3N may accelerate the wound healing process by inhibiting the activity of the serine proteases cathepsin G and GrB that are associated with inflammation, as well as matrix metalloproteinase 9 that is associated with extracellular matrix breakdown and remodeling (13,15,36). In addition, SA3N also inhibits T cell-derived leukocyte elastase, which helps attenuate neuropathic pain (14). Taken together, SA3N as a GrB activity inhibitor may serve various protective roles by acting on different targets. Thus, it was suggested that SA3N may serve its protective role on LPS-induced inflammatory response as the result of the concurrent effects of various factors and mechanisms.

In conclusion, the results of the present study indicated that GrB may be a potential pro-inflammatory factor that is induced by LPS, and the inhibition of GrB activity may block LPS-induced inflammatory response. It is hypothesized that GrB may be involved in the development of inflammation through regulation of the NF-κB pathway, mediated by TNF-α. Therefore, GrB has potential as a therapeutic agent for inflammatory diseases. However, the connection between GrB and TNF-α, and the molecular mechanism of their interaction requires further study.

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**Availability of data and material**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

JT and LW were responsible for the study concept and design. LW and SJ performed the western blot analysis. LX and LC performed the ELISA analysis. YZ performed the MTT assay. JT and LW assisted with data analysis and interpretation of results. LW drafted the manuscript. JT and LW provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved final version for publication.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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