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Sinomenine retards LPS-elicited inflammation via down-regulating CCAT1 in HaCaT cells

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ABSTRACT

Aims: The initiation of pressure ulcers is accompanied by inflammation. Sinomenine emerges as a potential anti-inflammation agent. The aim of this study was to corroborate its anti-inflammatory property in skin keratinocyte HaCaT cells. Long non-coding RNA colon cancer associated transcript-1 (CCAT1)-associated mechanisms were also investigated.

Main methods: HaCaT cells were stimulated with lipopolysaccharide (LPS) for 6 h after sinomenine pre-administration. Transfection was carried out to induce CCAT1 overexpression or silence it in HaCaT cells. Viability and apoptosis of HaCaT cells were determined by MMT and observed using flow cytometry, respectively. Protein expression was quantified using Western blot or ELISA. CCAT1 was measured by qRT-PCR.

Key findings: LPS notably decreased cell viability and exaggerated apoptosis with the cleavage of caspase-3/-9. The secretion of inflammatory factors was promoted. Sinomenine pre-administration maintained cell viability, blocked apoptosis and relieved inflammation with the decrease in cleaved caspase-3/-9 and inflammatory factors. LPS-induced phosphorylation of p65, IκBα and p38MAPK and overexpression of CCAT1 were precluded by sinomenine. CCAT1 overexpression, which per se induced inflammatory lesions, negated the positive effects of sinomenine with the restored phosphorylation of p65, IκBα, and p38MAPK.

Significance: Sinomenine played a protective role against LPS-induced inflammation. The anti-inflammatory activity of sinomenine might be mediated by CCAT1 down-regulation.

1. Introduction

Pressure ulcers are injuries in skin or underlying tissues over a bony prominence. It is a worldwide challenge affecting hospital and community patient populations [1]. Epidemiological evidences have identified that the primary causal factors for pressure ulcer aggravation include the decreased activity and mobility, perfusion and skin or pressure ulcer status [2]. Despite the etiology remains unclear, ischemia-reperfusion (IR)-caused damage is a causal factor [3]. A vast amount of findings have elucidated that IR exacerbates synthesis of reactive oxygen species (ROS) which induces over exuberant inflammation [4,5]. Moderate inflammation is normally driven to protect cells from adverse injuries, while over-inflammation or excessive inflammation generally causes disease [6]. A recent study suggested that reducing inflammation improves the wound healing of pressure ulcers [7]. Therefore, anti-inflammation agent may be a potential therapeutic strategy for promoting pressure ulcer healing.

Sinomenine is an alkaloid component extracted from Chinese medical plant *Sinomenium acutum* which has been clinically applied in treatment of rheumatoid arthritis and glomerular diseases [8]. Sinomenine has attracted increased attention as a potential anti-inflammation agent. It ameliorates IR-induced renal injury evidenced by preventing tubular cells against apoptosis, which proceeds with decrease in inflammation [9]. Its derivative sinomenine hydrochloride precludes epithelial to mesenchymal transition in breast cancer cells mainly through reducing the production of interleukin-6 (IL-6) [10]. In adjuvant-induced arthritis of rats, sinomenine can suppress inflammation, and alleviate joint damage [11]. More than that, its analgesic effect has been proved in rodents after inflammation and nerve injury [12]. Therefore, we investigated the role of sinomenine against inflammation in human skin keratinocyte HaCaT cells which are characterized by a highly preserved differentiation property [13].

Long non-coding RNA colon cancer associated transcript-1 (CCAT1) was firstly found to be transcribed off the cMYC enhancer in colon

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cancer [14]. In the stepwise studies, CCAT1 was found to be over-expressed in gastric cancer [15] and oral squamous cell carcinomas [16]. Strikingly, tumor necrosis factor alpha (TNF-α) administration was confirmed to induce the generation of CCAT1 in caco-2 cells, implying that CCAT1 is associated with the inflammatory cascades [17]. Moreover, a recent study showed that CCAT1 regulates pro-inflammatory response in intestinal epithelial cells [18]. Nevertheless, it still remains to be further validated whether CCAT1 is implicated in the initiation of inflammation in pressure ulcers. In addition, it is worth exploring whether the anti-inflammatory activity of sinomenine is mediated by CCAT1.

Here we set out to corroborate an anti-inflammatory property of sinomenine in skin keratinocyte HaCaT cells which exhibit a prompt inflammation in response to adverse stimulation [19,20]. Moreover, HaCaT cells are typical of highly preserved differentiation capacity, which contributes to its general application as a paradigm for skin keratinocytes in vitro [13]. In addition, we investigated whether this anti-inflammatory activity of sinomenine is associated with CCAT1, and we also ascertained the role of CCAT1 in inflammation. Besides, we further dissected whether key signaling transduction cascades are implicated in this process.

2. Materials and methods

2.1. Cell culture and sinomenine administration

Human skin keratinocyte HaCaT cells were obtained from Cell Line Service (Order No. 300493) (Eppelheim, Germany). According to information from the supplier, HaCaT cells were in vitro spontaneously transformed keratinocytes from histologically human normal skin. HaCaT cells are wildly used for studies before in vivo translation. HaCaT cells were cultured in Dulbecco's modified Eagle medium (American Type Culture Collection, ATCC, Rockville, MD, USA) in addition with 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Invitrogen), 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 units of penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Sinomenine was dissolved in dimethylsulfoxide (Sigma-Aldrich) and then diluted into various concentrations (0.1–2.0 μM) using medium. The cells were pre-administrated with sinomenine for 24 h before exposed to LPS (Sigma-Aldrich) (0–10 μg/mL) for 6 h.

2.2. Transfection

CCAT1 was cloned into pcDNA3.1 plasmid (Invitrogen), and HaCaT cells were transfected with pcDNA3.1-CCAT1 in the presence of lipofectamine 3000 reagent (Invitrogen). Stably expressed CCAT1 cells were selected using G418 (Invitrogen). To induce the silence of CCAT1, Silencer siRNA construction kit (Ambion, Huntingdo, UK) was applied to design and synthesize siRNA targeting CCAT1 (si-CCAT1). Next, si-CCAT1 was transfected into HaCaT cells using lipofectamine 3000. Transduction efficiency was identified using quantitative reverse transcription-PCR (qRT-PCR).

2.3. Cell viability detection

Cell viability was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay kit (Beyotime, Shanghai, China). In brief, HaCaT cells were seeded into each well of 6-well plate in a density of 1×10^6 cells per well. Next, HaCaT cells were pre-treated with sinomenine and incubated for 24 h. Next, the cells were exposed to LPS for 6 h. Then, 1 mL MTT reagent in a concentration of 1 mg per mL was supplied into each well and cultured with the cells for 2 h at 37 °C. Finally, the absorbance was detected at 540 nm using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Apoptosis examination

To assess apoptosis process, Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit from Abcam (Cambridge, UK) was used. In short, 500,000 cells were collected, trypsinized using trypsin (Pierce, Appleton, WI, USA) and re-suspended in 500 μL binding buffer. Then, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added. Continually, the culture was maintained for 5 min at room temperature in the dark. Finally, the cells were observed using a CytoFLEX Flow Cytometer (Beckman Coulter, IN, USA). Annexin V-FITC-positive and PI-negative cells were identified as apoptotic cells. The results were depicted as the percentage of Annexin V-FITC-positive and PI-negative cells.

2.5. qRT-PCR

Total RNA was extracted using a Trizol reagent kit (Invitrogen) according to manufacturer's protocol. CCAT1 was analyzed by qRT-PCR according to a previous reported method [21]. In short, cDNA was synthesized with random primers. qRT-PCR was performed using the specific primers and probes (Thermo Fisher Scientific). GAPDH served as an endogenous control. Relative quantification was calculated using △Ct method.

2.6. Enzyme-linked immuno sorbent assay (ELISA)

ELISA was performed using kit from R&D systems (Abingdon, UK). Briefly, a 96-well plate was coated with buffer supplemented with antibodies against IL-6 and TNF-α, and then the culture was maintained overnight at 4 °C. Next, the culture was blocked using assay diluent at room temperature for 1 h after washed using PBS. Subsequently, the cell supernatant and standards of IL-6 and TNF-α were added into the plate and cultured for 2 h at room temperature. After washed, the culture was incubated with the detection antibody for 1 h at room temperature. Continually, the culture was incubated with enzyme for 30 min and substrate solution for 15 min in the dark. Finally, the reaction was stopped by the stop solution, and the absorbance was read at 450 nm.

2.7. Western blotting

Whole proteins from the cells were extracted using RIPA lysis buffer (Beyotime) in addition with protease inhibitors (Roche Applied Science, Indianapolis, USA). BCA™ kit (Pierce) was used to examine the concentration of obtained protein extract which was then separated on a Bio-Rad system (Bio-Rad, Hercules, CA, USA), and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Next, the membrane strips were incubated with anti-caspase-3 (14220) (Cell Signaling Technology, CST, Danvers, MA, USA), anti-caspase-9 (9508) (CST), anti-IL-6 (12153) (CST), anti-TNF-α (orb239747) (Biorbyt, Cambridge, UK), anti-cyclooxygenase 2 (Cox-2) (ab179800) (Abcam), anti-inducible nitric oxide synthase (iNOS) (orb500991) (Biorbyt), anti-nuclear factor-kappa B p65 (NF-κB p65) (MBS839188) (MyBioSource, California, USA), anti-phospho-Ser276 NF-κB p65 (MBS001656) (MyBioSource), anti-inhibitor of nuclear factor kappa-B alpha (IκBα) (MBS222320), anti-phospho-Tyr305 IκBα (MBS854130) (MyBioSource), anti-p38 mitogen-activated protein kinase (p38MAPK) (orb14630) (Biorbyt), anti-phospho-Tyr182 p38MAPK (orb14942) (Biorbyt) and anti-β-actin (4967) (CST) antibodies (1:1,000) at 4 °C overnight. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (7074) (CST) and rabbit anti-mouse IgG (58802) (CST) were applied to probe the antibody-targeting protein complexes in a dilution of 1:5,000 at room temperature for 1 h. Finally, enhanced chemiluminescence Western blotting substrate (Millipore) was added as

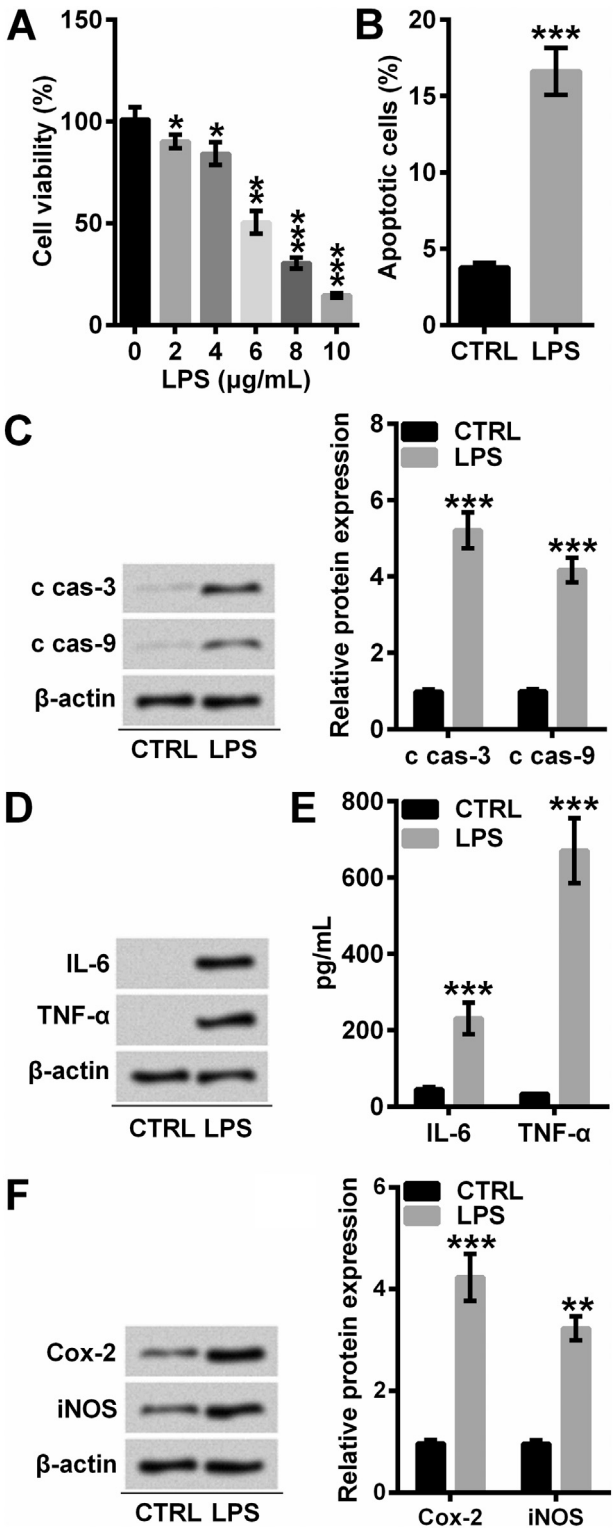

Fig. 1. Effect of lipopolysaccharide (LPS) on cell viability, apoptosis process, and inflammatory factor production in HaCaT cells. (A) HaCaT cells were tested in the presence of LPS. The cells were induced with the indicated concentrations of LPS. Viability of HaCaT cells by MTT method. HaCaT cells were administrated with 6 μg/mL LPS. (B) Apoptosis process by observation using flow cytometry. (C) Apoptosis-associated proteins, cleaved caspase-3 (c cas-3) and c cas-9, by Western blotting. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) concentration by (D) Western blotting and (E) ELISA. (F) Cyclooxygenase (Cox-2) and inducible nitric oxide synthase (iNOS) by Western blotting. All values are depicted as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to suggest significant difference to control

described by the manufacturer's protocol. The protein signaling was captured and quantified using Bio-Rad ChemiDoc™ XRS system and Image Lab™ Software (Bio-Rad).

2.8. Data analysis

Data were expressed as mean ± standard deviation. Significance was evaluated using a Student's *t*-test and one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. Probability values of $P < 0.05$ were considered to represent a significant difference.

3. Results

3.1. Effects of LPS on viability, apoptosis and inflammatory factor accumulation in HaCaT cells

First of all, we evaluated the effect of LPS on viability of HaCaT cells using MTT. As suggested in Fig. 1A, LPS exerted a visible cytotoxic effect at the indicated concentrations ($P < 0.05$, $P < 0.01$ and $P < 0.001$). Given that LPS in a concentration of 6 μg/mL caused a decrease of appropriately 50% in cell viability, we next treated HaCaT cells with 6 μg/mL LPS. Notably, LPS resulted in a significant exacerbation in apoptosis ($P < 0.001$) (Fig. 1B), which was further indicated by the increase in cleavage of caspase-3 and caspase-9 (both $P < 0.001$) (Fig. 1C). Next, we investigated whether LPS stimulates inflammatory factor production in HaCaT cells. As shown in Fig. 1D and E, LPS significantly facilitated IL-6 and TNF-α (both $P < 0.001$) excretion, with overproduction of Cox-2 ($P < 0.001$) and iNOS ($P < 0.01$) (Fig. 1F). Collectively, LPS efficiently introduced an inflammatory reaction in HaCaT cells.

3.2. Sinomenine suppressed the over-inflammatory in LPS-treated HaCaT cells

To evaluate the anti-inflammatory property of sinomenine, we firstly examined the role of sinomenine in viability of HaCaT cells. As presented in Fig. 2A, sinomenine demonstrated a non-significant cytotoxic effect at a lower concentration (0.1–1.0 μM) ($P > 0.05$), while a visible decrement in cell viability was induced by sinomenine with a concentration of 2.0 μM ($P < 0.05$). By contrast, the viability of LPS-treated HaCaT cells was does-dependently repressed by sinomenine ($P < 0.05$) (Fig. 2B). Consequently, we pre-administrated HaCaT cells with 1.0 μM sinomenine before LPS stimulation. Consistently, LPS-induced apoptosis was evidently ($P < 0.05$) blocked by sinomenine (Fig. 2C), with the decrease in cleavage of caspase-3 ($P < 0.01$) and caspase-9 ($P < 0.05$) (Fig. 2D). Notably, sinomenine administration resulted in a down-regulation in IL-6 ($P < 0.01$), TNF-α ($P < 0.01$) (Fig. 2E and F), Cox-2 ($P < 0.01$) and iNOS ($P < 0.05$) (Fig. 2G). Taken together, sinomenine exhibited an anti-inflammatory property in LPS-treated HaCaT cells.

3.3. Sinomenine blocked LPS-evoked activation of NF-κB and MAPK

In our study, the effect of LPS on NF-κB and MAPK cascades was explored. As shown in Fig. 3A, LPS caused a noted phosphorylation of p65 at Ser276 ($P < 0.001$) and IκBα at Tyr305 ($P < 0.01$), while the phosphorylated biosynthesis of p65 ($P < 0.01$) and IκBα ($P < 0.05$) was prominently suppressed by sinomenine (Fig. 3A). Conspicuously, LPS facilitated ($P < 0.01$) the phosphorylation of p38MAPK at Tyr182. However, this phosphorylation was apparently ($P < 0.05$) blunted by sinomenine administration (Fig. 3B). Therefore, it can be concluded that sinomenine blocked the activation of NF-κB and MAPK triggered by LPS.

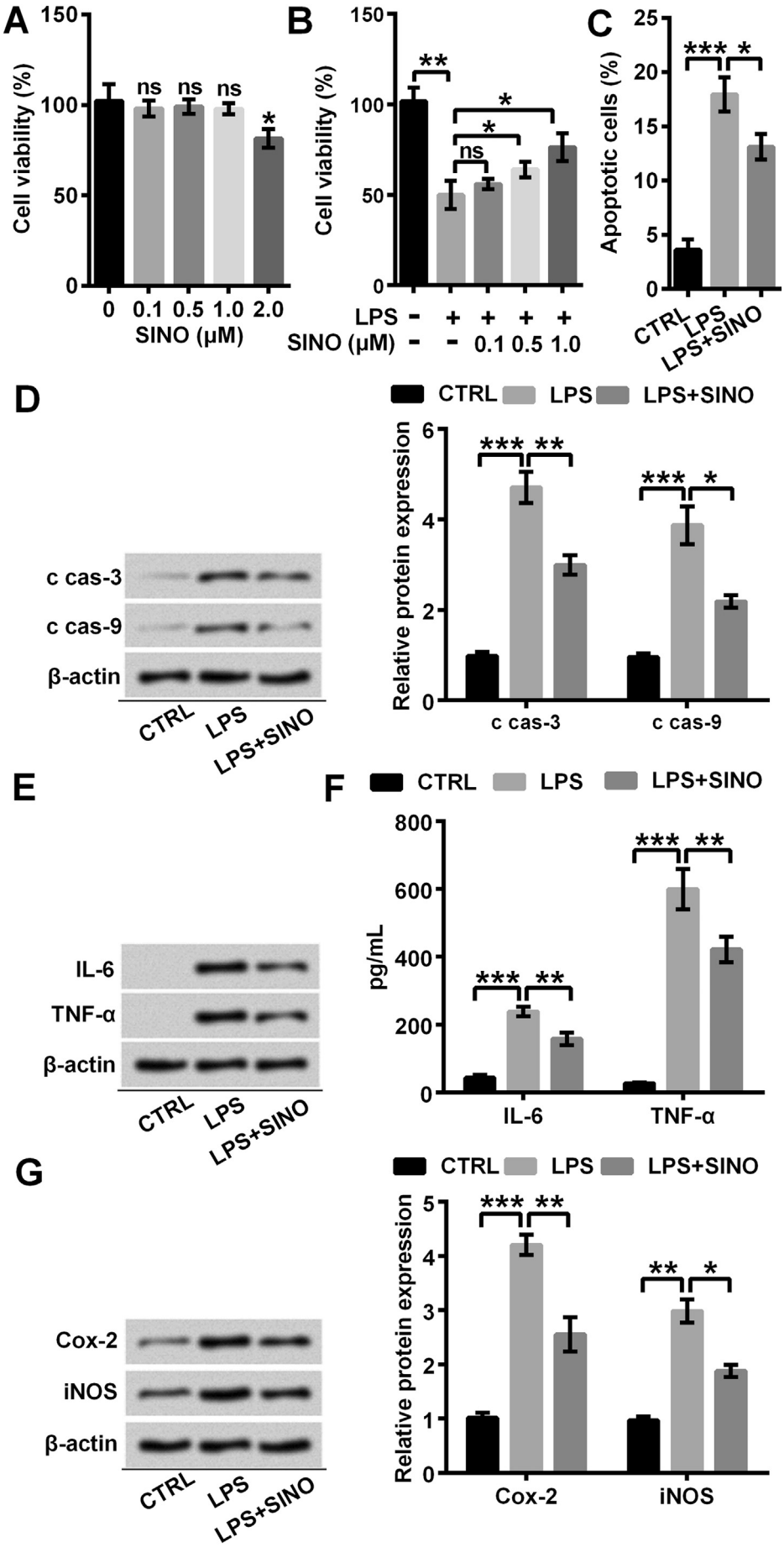

Fig. 2. Effect of sinomenine (SINO) on cell viability, apoptosis process, and inflammatory factor production in lipopolysaccharide (LPS)-treated HaCaT cells. (A) HaCaT cells were tested in the presence SINO. The cells were induced with the indicated concentrations of SINO. Viability of HaCaT cells by MTT method. HaCaT cells were administrated with 1 μM SINO for 24 h before treated with LPS. (B) Viability of HaCaT cells by MTT. (C) Apoptosis process by observation using flow cytometry. (D) Apoptosis-associated proteins, cleaved caspase-3 (c cas-3) and c cas-9, by Western blotting. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) concentration by (E) Western blotting and (F) ELISA. (G) Cyclooxygenase (Cox-2) and inducible nitric oxide synthase (iNOS) by Western blotting. All values are depicted as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to suggest significant difference to control group. $^{ns}P > 0.05$.

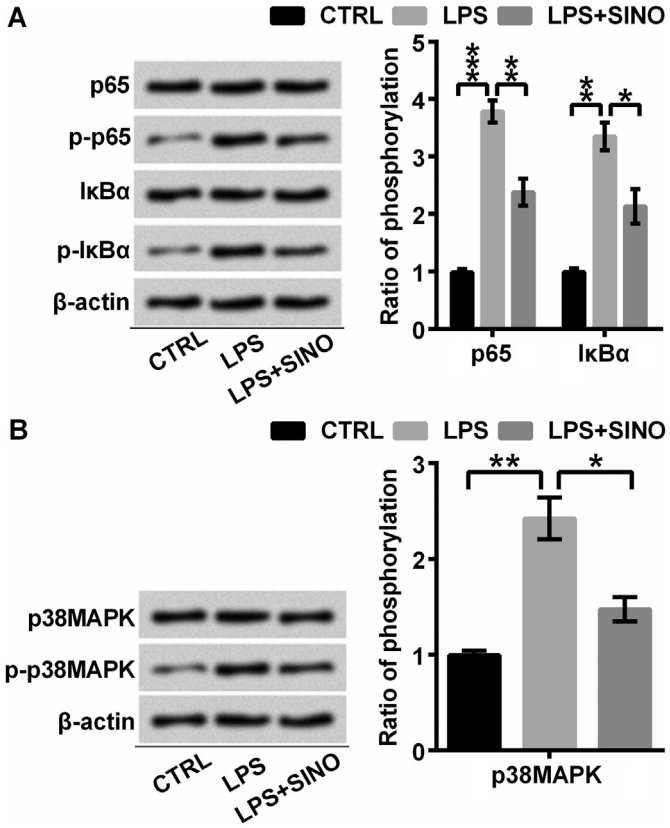

Fig. 3. Effects of sinomenine (SINO) on protein expression of NF-κB p65, phospho-Ser276 NF-κB p65, IκBα, phospho-Tyr305 IκBα, p38MAPK and phospho-Tyr182 p38MAPK in lipopolysaccharide (LPS)-treated HaCaT cells. HaCaT cells were administrated with 1 μM SINO for 24 h before treated with 6 μg/mL LPS for 6 h. (A) NF-κB p65 (60 kDa), phospho-Ser276 NF-κB p65 (60 kDa), IκBα (36 kDa), phospho-Tyr305 IκBα (36 kDa), (B) p38 MAPK (38 kDa) and phospho-Tyr182 p38MAPK (38 kDa) by Western blotting. Phosphorylated ratio is represented after normalization to β-actin. All values are depicted as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to suggest significant difference to control or LPS groups.

3.4. CCAT1 upregulation contributed to inflammatory damages of HaCaT cells

To better understand the modulatory role of CCAT1 in inflammation, we established CCAT1-overexpressed ($P < 0.001$) and -deficient ($P < 0.01$) HaCaT cells (Fig. 4A). Next, the cells were subjected to a cascade of assays for viability, apoptosis, and protein expression. We ascertained CCAT1 resulted in a significant decrease ($P < 0.05$) in viability, while its silence reversely enhanced ($P < 0.05$) the viability of HaCaT cells (Fig. 4B). Further, CCAT1 initiated apoptosis process which was evidenced by the increased number of apoptotic cells ($P < 0.01$) (Fig. 4C) and abundance of cleaved caspase-3 and caspase-9 (both $P < 0.001$) (Fig. 4D). By contrast, CCAT1 knockdown exerted a not significant effect on apoptosis ($P > 0.05$) (Fig. 4C–D). Additionally, CCAT1 overexpression was accountable for the expression and secretion of IL-6 ($P < 0.01$) and TNF-α ($P < 0.001$), while CCAT1 silence conferred a negligible role on inflammatory factors ($P > 0.05$) (Fig. 4E–F). Moreover, CCAT1 resulted in the abundance of Cox-2 and iNOS (both $P < 0.05$) in HaCaT cells whereas CCAT1-deficient cells showed a not significant alteration in the expression of Cox-2 and iNOS ($P > 0.05$) (Fig. 4G). Summarily, we considered that CCAT1 upregulation evoked an evident inflammatory damage in HaCaT cells while its silence showed a not significant effect on inflammation.

3.5. Sinomenine exhibited an anti-inflammatory activity through down-regulating CCAT1

Of particular importance, LPS-treated HaCaT cells showed an obvious increment in CCAT1 expression ($P < 0.001$) (Fig. 5A). Besides, a notable reduction in CCAT1 was caused by sinomenine pre-administration ($P < 0.01$) before LPS stimulation (Fig. 5A).

Based on these results, we hypothesized that LPS-triggered over-inflammation might be dependent on CCAT1, and sinomenine might inhibit inflammation by down-regulating CCAT1. To confirm our assumption, stably expressing CCAT1 HaCaT cells ($P < 0.001$) (Fig. 4A) were subjected to sinomenine incubation, followed by LPS stimulation. Interestingly, CCAT1 overexpression caused an obvious reduction ($P < 0.05$) in cell viability (Fig. 5B), facilitated ($P < 0.05$) apoptosis process (Fig. 5C) with the accumulation of cleaved caspase-3 and caspase-9 (both $P < 0.01$) (Fig. 5D). Additionally, CCAT1 promoted the secretion and production of IL-6 and TNF-α (both $P < 0.01$) (Fig. 5E and F), which was accompanied by the accumulation of Cox-2 ($P < 0.01$) and iNOS ($P < 0.05$) (Fig. 5G).

Consistently, the phosphorylation of p65 ($P < 0.01$), IκBα ($P < 0.05$) (Fig. 6A) and p38MAPK ($P < 0.05$) was restored by CCAT1 (Fig. 6B). Summarily, sinomenine exhibited an anti-inflammatory activity by retarding the production of CCAT1 with the blockage of NF-κB and MAPK cascades.

4. Discussion

Skin wound healing process physiologically consists of four dynamic overlapping stages, including hemostasis, inflammation, proliferation and remodeling [22]. Excessive inflammation retards the process of wound repairing [23]. Inflammation disorder always exits in pressure ulcer [24]. Though the exact mechanism by which inflammation occurs in pressure ulcer is not fully elucidated, inhibition of over-inflammation probably exerts a role in protection of HaCaT cells against injuries [25].

The results of this study suggested that viability of HaCaT cells can be obviously maintained by sinomenine administration before LPS stimulation. Moreover, IL-6, TNF-α, Cox-2, and iNOS levels were notably repressed. These findings had pivotal implications as previous reports revealed that pressure induces the production of IL-6 and TNF-α which contributes to the formation of pressure ulcer [26]. Additionally, celecoxib, a selective Cox-2 inhibitor, improves the wound healing of pressure ulcers, mainly through decreasing the generation of iNOS and Cox-2 [7]. In chemical hypoxia-caused cytotoxicity and inflammation, a crucial role has been ascribed to the ability of hydrogen sulfide to block the activation of ROS/NF-κB/Cox-2 signaling cascade [27]. Indeed, the anti-inflammatory effects of sinomenine in diseases, such as neuro-inflammation [28], traumatic spinal cord injury [29] and obstructive nephropathy [30], have been reported.

Our findings were in consistent with Yang et al. who observed that administration of sinomenine reduces cytokines release and blunts NF-κB pathway activation in intracerebral hemorrhage mice [31]. NF-κB is implicated in the modulation of pro-inflammatory genes encoding cytokine and chemokine [32]. Substantial evidences revealed that pro-inflammatory stimuli-activated MAPK cascade post-transcriptionally modulates the biosynthesis of pro-inflammatory proteins [33]. Intriguingly, sinomenine hydrochloride-induced apoptosis is indeed dependent on the activation of MAPK cascade in breast cancer cells [34], which implies that MAPK cascade might participate in the progress. Here we showed that LPS-elicited p38MAPK phosphorylation was significantly decreased by sinomenine. Collectively, we considered that sinomenine relieved the excessive inflammatory reaction with blockage of NF-κB and MAPK signaling transduction cascades.

Alaiyan et al. reported that CCAT1 level is high in colonic tissues from individuals with benign colonic disorders, and the up-regulation of CCAT1 is more prominent in the patients with inflammatory conditions or severe colonic inflammation [35], suggesting that CCAT1 might

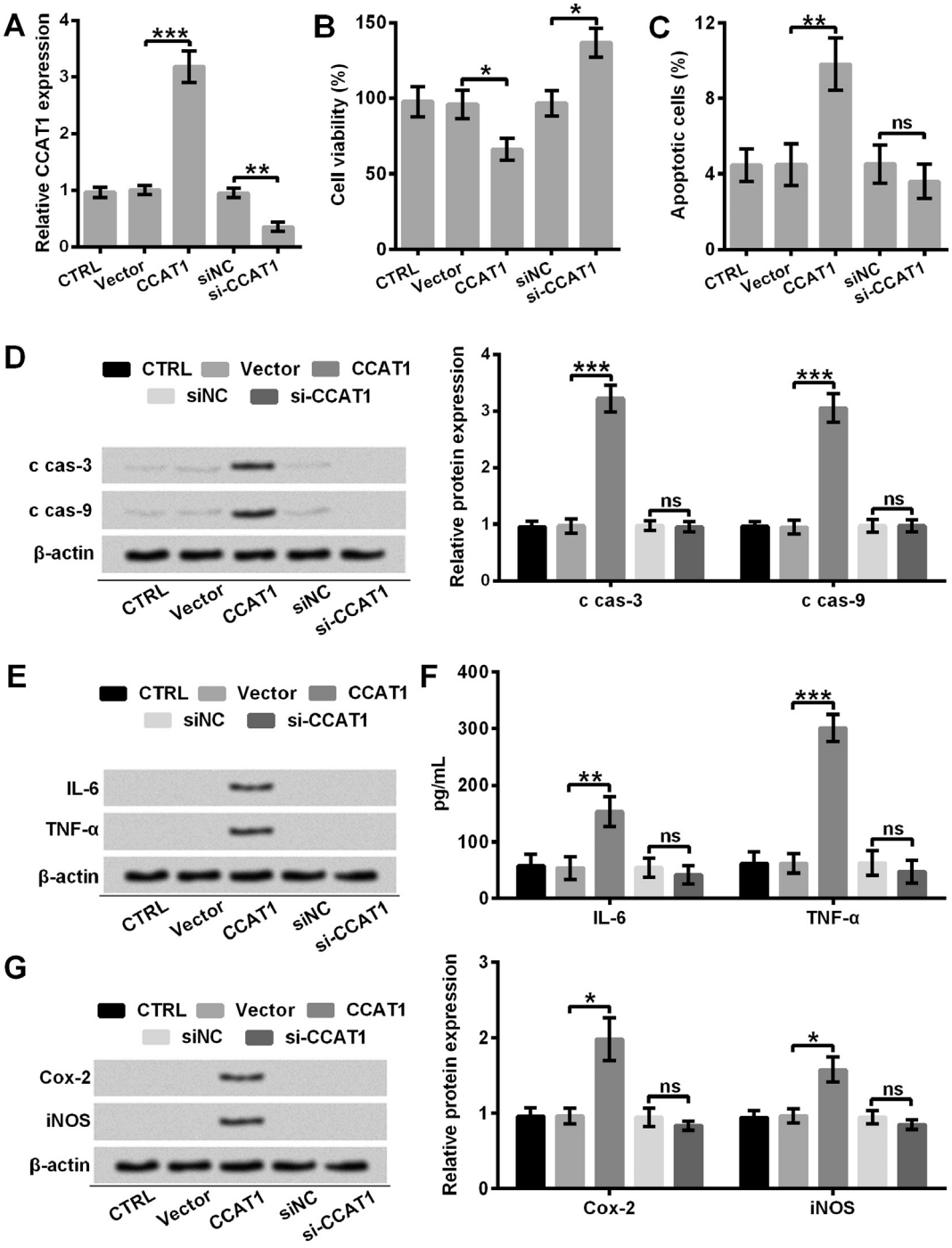

Fig. 4. CCAT1 overexpression was included in induction of inflammatory lesions of HaCaT cells. (A) Total mRNA was extracted and CCAT1 level was analyzed by qRT-PCR after transfection. Relative expression was normalized by GAPDH. (B) Viability of HaCaT cells by MTT. (C) Apoptosis process by observation using flow cytometry. (D) Apoptosis-associated proteins, cleaved caspase-3 (c cas-3) and c cas-9, by Western blotting. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) production by (E) Western blotting and (F) ELISA. (G) Cyclooxygenase (Cox-2) and inducible nitric oxide synthase (iNOS) by Western blotting. Data are repressed as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ were considered to suggest significant difference to vector or siNC. $^{ns}P > 0.05$.

be implicated in inflammation reaction. In malignancies, CCAT1 emerges as a competing endogenous RNA and competitively targets common microRNAs (miRNA) which post-transcriptionally target transcripts [36]. These notions were in line with our findings that CCAT1 initiated inflammatory insults in HaCaT cells. However, the mechanism through which CCAT1 mediates inflammation remains largely unknown. Despite that sinomenine has been found to regulate the biosynthesis of miRNAs [37,38], with regard to another non-coding RNA lncRNAs, the available information is limited. Importantly, we firstly reported that sinomenine conferred a suppressive effect on the expression of CCAT1 induced by LPS.

Of particular interest is that CCAT1 is found closely associated with malignancies via inflammation-related signaling pathways such as Wnt [39] and MAPK [40]. These signaling pathways not only participate in the proliferation and apoptosis processes [41,42], but also mediate the inflammation response [43,44]. We found the protective effects and

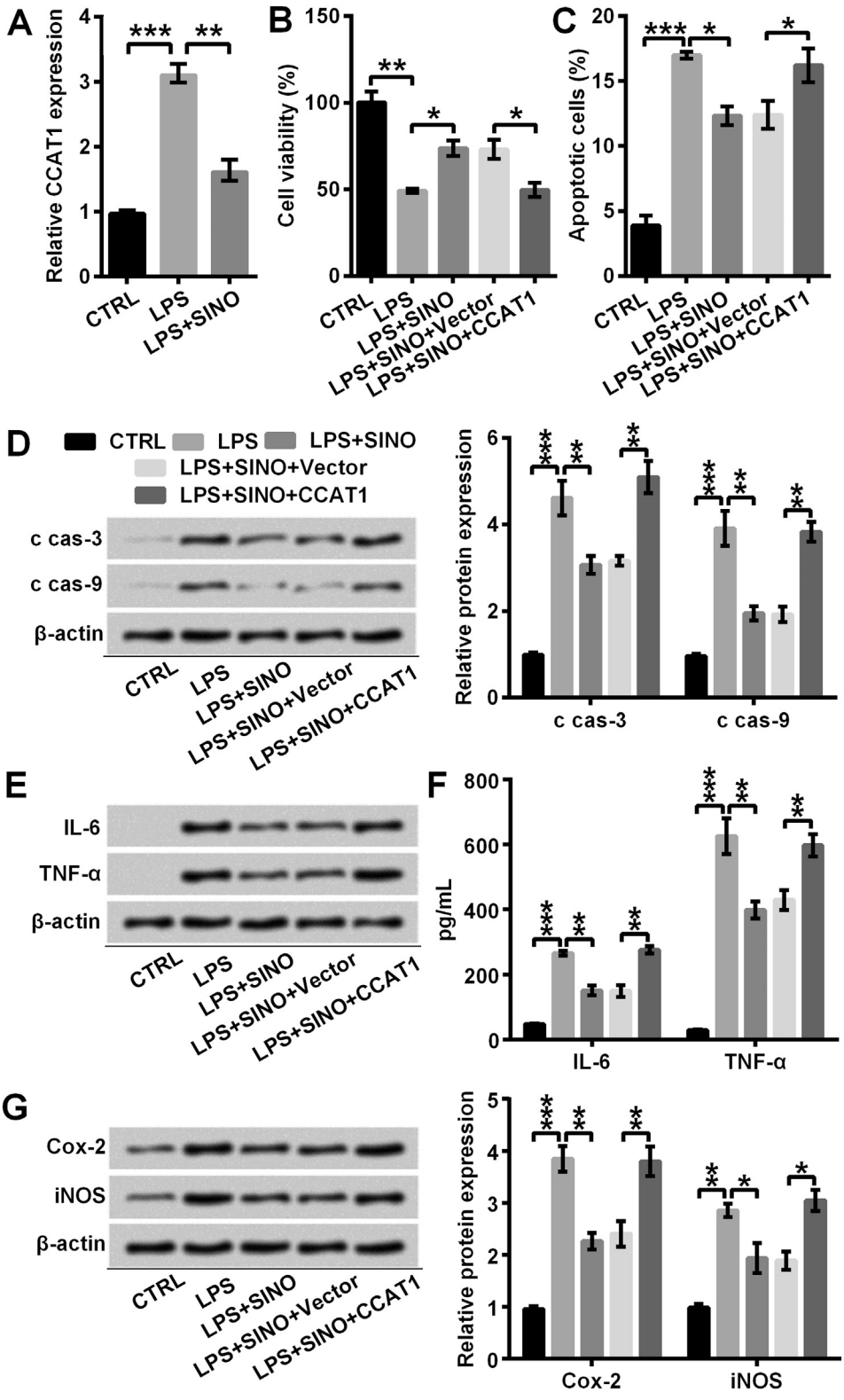

Fig. 5. Effects of CCAT1 on the protective property of sinomenine (SINO) in lipopolysaccharide (LPS)-treated HaCaT cells. (A) CCAT1 expression was detected by qRT-PCR after HaCaT cells were pretreated by 1 μM SINO (24 h) prior to LPS stimulation. Stably expressing CCAT1 cells were administrated with 1 μM SINO for 24 h before treated with LPS. (B) Viability of HaCaT cells by MTT. (C) Apoptosis process by observation using flow cytometry. (D) Apoptosis-associated proteins, cleaved caspase-3 (c cas-3) and c cas-9, by Western blotting. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) production by (E) Western blotting and (F) ELISA. (G) Cyclooxygenase (Cox-2) and inducible nitric oxide synthase (iNOS) by Western blotting. All values are depicted as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to suggest significant difference to control, LPS, or vector groups. $^{ns}P > 0.05$.

anti-inflammatory properties of sinomenine were negated in CCAT1-overexpressed HaCaT cells, suggesting that sinomenine had effects in dependence on the suppression of CCAT1. In addition, the activation of NF-κB and MAPK pathways was triggered. These two cascades are involved in inflammation and inflammation-associated diseases in a crosstalk manner [45]. Similarly, through modulating NF-κB, other alkaloid like vinca contributes to the apoptosis in human tumor cells [46].

In summary, we consolidated that sinomenine played a protective role against LPS-induced inflammation, suggesting that sinomenine might be a new therapeutic option for pressure ulcer. Sinomenine-mediated down-regulation of CCAT1 might contribute to the anti-inflammation activity. Molecularly, the bluntness of NF-κB and MAPK cascades might be dependent on sinomenine-caused suppression of CCAT1.

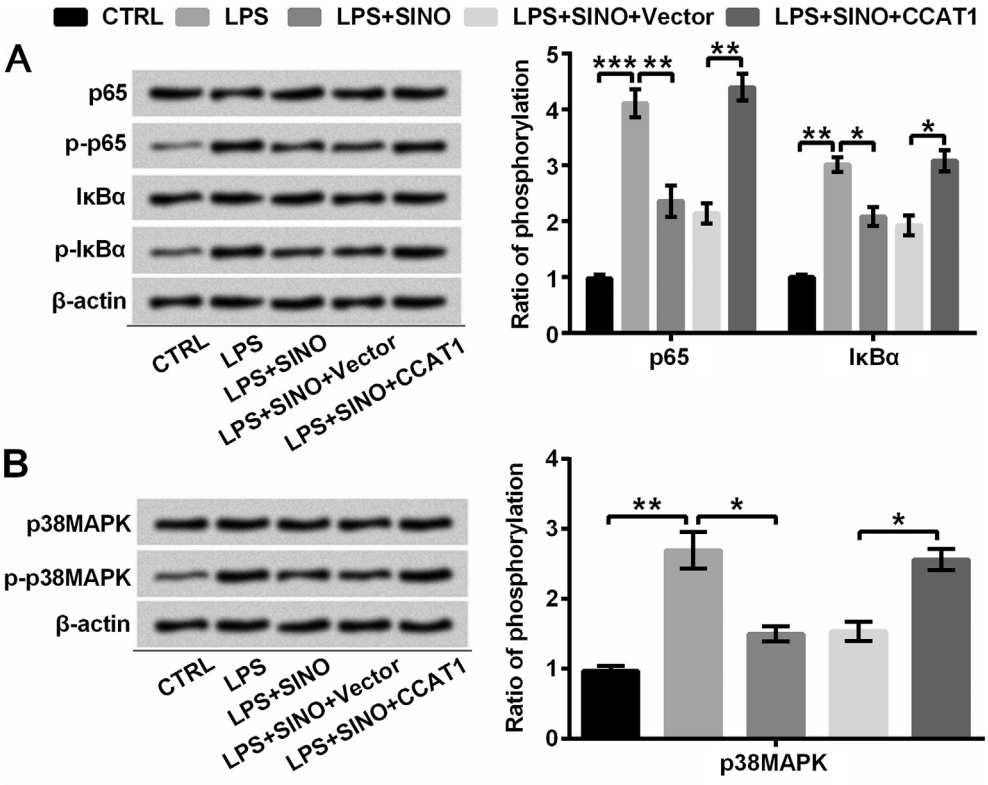

Fig. 6. Function of CCAT1 on NF-κB and MAPK cascades in sinomenine (SINO)-pre-incubated and lipopolysaccharide (LPS)-treated HaCaT cells. Stably expressing CCAT1 HaCaT cells were administrated with 1 μM SINO for 24 h before treated with 6 μg/mL LPS for 6 h. (A) NF-κB p65 (60 kDa), phospho-Ser276 NF-κB p65 (60 kDa), IκBα (36 kDa), phospho-Tyr305 IκBα (36 kDa), (B) p38 MAPK (38 kDa) and phospho-Tyr182 p38MAPK (38 kDa) by Western blotting. Phosphorylated ratio is re-presented after normalization to β-actin. All values are depicted as the mean ± standard deviation of three independent experiments. Values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to suggest significant difference to control, LPS, or vector groups.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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