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Siwen Hui
Xianning Medical College: Hubei University of Science and Technology Faculty of Medicine
Wen Kan
5th Medical Center of Chinese PLA General Hospital
Shuanglin Qin
Xianning Medical College: Hubei University of Science and Technology Faculty of Medicine
Ping He
5th Medical Center of Chinese PLA General Hospital
Jia Zhao
5th Medical Center of Chinese PLA General Hospital
Hui Li
5th Medical Center of Chinese PLA General Hospital
Jun Bai
General Hospital of People's Liberation Army: Chinese PLA General Hospital
Jincai Wen
5th Medical Center of Chinese PLA General Hospital
Wenqing Mou
5th Medical Center of Chinese PLA General Hospital
Manting Hou
5th Medical Center of Chinese PLA General Hospital
Ziying Wei
5th Medical Center of Chinese PLA General Hospital
Xiaohe Xiao
5th Medical Center of Chinese PLA General Hospital
Guang Xu
Capital Medical University School of Traditional Chinese Medicine
Zhaofang Bai (baizf2008@hotmail.com)
5th Medical Center of Chinese PLA General Hospital https://orcid.org/0000-0002-6208-150X

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Glycyrrhiza Uralensis Polysaccharides Inhibits the cGAS-STING Signaling Pathway and Ameliorates Cecum Ligation and Puncture-induced Sepsis

Siwen Hui[1,2,3,#], Wen Kan[2,3,#], Shuanglin Qin[1,2,3,#], Ping He[2,3,#], Jia Zhao[2,3], Hui Li[2,3], Jun bai[5], Jincai Wen[2,3], Wenqing Mou[2,3], Manting Hou[2,3], Ziying Wei[2,3], Xiaohe Xiao[2,3,*], Guang Xu[2,3,4,*], Zhaofang Bai[2,3,*]

[1]School of Pharmacy, Xianning Medical College, Hubei University of Science and Technology, Xianning, PR China.

[2]Department of Hepatology, the Fifth Medical Center of Chinese PLA General Hospital, Beijing, 100039, China.

[3]China Military Institute of Chinese Materia, Fifth Medical Center of Chinese PLA General Hospital, Beijing, 100039, China.

[4]School of Traditional Chinese Medicine, Capital Medical University, Beijing, 100069, China.

[5]Department of Neurosurgery, General Hospital of Chinese People Liberty Army.

[#]These authors contribute equally to the work.

***Correspondence to:**

[2,3]Zhaofang Bai, Department of Hepatology, the Fifth Medical Center of PLA General Hospital, Beijing, 100039, China. Email: baizf2008@hotmail.com.

[2,3,5]Guang Xu, School of Traditional Chinese Medicine, Capital Medical University, Beijing, 100069, China. Email: guang_xu@ccmu.edu.cn.

[2,3]Xiaohe Xiao, Department of Hepatology, the Fifth Medical Center of PLA General Hospital, Beijing, 100039, China. Email: pharmacy_302@126.com.

Abbreviations

c-di-AMP	cyclic dimeric adenosine monophosphate	**ELISA**	Enzyme-linked immunosorbent assay
diABZI	trihydrochloride	**DMEM**	Dulbecco's modified Eagle's medium
DMXAA	5,6-dimethylxanthenone-4-acetic acid	**hPBMCs**	human peripheral blood mononuclear cells
c-di-IMP	cyclic dimeric inosine monophosphate	**IRF3**	interferon regulatory factor 3
c-di-GMP	cyclic dimeric guanosine monophosphat	**TBK1**	TANK binding kinase 1
TNF-α	tumor necrosis factor-α	**cGAMP**	Cyclic guanosine monophosphate–adenosine monophosphate
IL-6	interleukin-6	**CXCL10**	C-X-C motif chemokine ligand 10

Abstract

Sepsis is a disease of multiple organ dysfunction caused by infection. It threatens human health and is one of the major human mortality problems. Recent researches have shown that cGAS-STING pathway is involved in the development and progression of sepsis. Here, we report that Glycyrrhiza Uralensis Polysaccharides

(GUP), one of the major components of licorice, has a protective effect against cecal ligation and puncture (CLP) sepsis in mice. We found that GUP reduced pathological changes in CLP sepsis-inducing tissues and the expression of genes downstream (IFN-β and TNF-α) of cGAS-STING pathway. Moreover, GUP significantly reduced the expression of IFN-β, IL-6 and TNF-α mediated by a mouse-specific activators of STING (DMXAA) in mice. In vitro studies, GUP inhibited the activation of the cGAS-STING pathway as evidenced by inhibition of the expression of related downstream genes, such as IFN-β, IL-6, TNF-α and interferon-stimulated genes (CXCL10). Mechanistically, GUP inhibited the activation of cGAS-STING pathway by inhibiting the interaction of STING with TBK1 and TBK1 with IRF3. In conclusion, our studies suggest that GUP is an effective inhibitor of the cGAS-STING pathway, which may be a potential medicine for the treatment of inflammatory diseases mediated by the cGAS-STING pathway.

Keywords

Glycyrrhiza uralensis polysaccharides; cGAS-STING pathway; sepsis; cecum ligation and puncture sepsis

Introduction

Sepsis is a systemic inflammatory response resulting from a bacterial or fungal infection, Life-threatening multiple organ dysfunction developed as the disease progresses[1]. Sepsis and septic shock are major healthcare problems, affecting millions of patients worldwide each year. It has become a top ten leading cause of morbidity and mortality[2]. Despite excellent success achieved in early detection and

emergency response management, sepsis remains an important factor in the death of infected patients[3, 4].

cGAMP synthase (cGAS) can be activated by exogenous DNA, DNA released by damaged cells and mitochondrial leakage of DNA as well as by intracellularly production of micronuclei, catalyzing the creation of second messenger cGAMP and stimulating STING signaling[5, 6]. STING then moves from the endoplasmic reticulum to the Golgi apparatus, reaching the perinuclear endosome before interacting with TNBK-binding kinase-1 (TBK1)[7]. TBK1 then phosphorylates STING, IRF3 and TRAF6, as well as its self, resulting in the expression of type I IFNs and other inflammatory cytokines[5, 8]. A growing number of researches have shown that the immoderate activation of cGAS-STING pathway could trigger a range of inflammatory responses and autoimmune diseases, including alcoholic liver disease[9], ischemic brain injury[10], rheumatoid arthritis[11] and myocardial infarction[12]. At the same time, the cGAS-STING pathway also plays a significant role in sepsis and over-activation of this signaling pathway is a pathogenic event in sepsis[13, 14].

Licorice is an ancient and widely-used herb, which has been recorded in many national pharmacopoeias. It is used in various herbal prescriptions[15]. Licorice has such functions as anti-cancer[16], anti-inflammatory[17-20], liver protection[21] and other pharmacological activities[22]. Recent studies depicted that polysaccharides and other licorice extracts are the main active components with strong biological and pharmacological activities[23]. Polysaccharides are naturally occurring polymeric

compounds consisting of a variety of monosaccharides bonded by glycosidic bonds and have been found to have a variety of diverse and powerful physiological effects [24]. Studies have shown that polysaccharides have greater development value and application potential in anti-inflammatory. For example, astragalus polysaccharides reduces gouty arthritis[25], lentinan has a good inhibitory effect on neuroinflammation[26]. As one of the main components of licorice, GUP has not only enhanced immunomodulatory, anti-tumor and antioxidant effects[27-29], but also exhibit anti-inflammatory effects[30]. Although the anti-inflammatory activity of GUP has been established, the exact mechanism of action and molecular targets remain unknown.

In our study, we aim to explore the role of GUP in the treatment of inflammation, particularly in the cGAS-STING pathway-mediated CLP sepsis, and to explore the potential mechanisms of its therapeutic role. We found that GUP inhibited the activation of the cGAS-STING pathway by inhibiting the interaction of STING with TBK1 and TBK1 with IRF3, thereby reducing the harm of sepsis.

METHODS DETAILS

2.1 Reagents

GUP (WH-0960) was gained from Shanghai Ronghe Medical Technology Development Co, LTD. Its purity was ≥95.0% (purity values are presented by the company) and the raw materials of GUP (licorice) was purchased from Shanghai Wanshicheng Sinopod Products Co, LTD. Anti-α-tubulin (66031-1-Ig, PTG), anti-Lamin B1 (66095-1-Ig, PTG), anti-IRF3 (11312-1-AP, PTG), anti-HSP90 (13171-1-

AP, PTG), anti-hp-IRF3 (ab76493, abcam), anti-STING (19851-1-AP, PTG), anti-mp-IRF3 (GTX86691, GTX), anti-HA Tag (66006-2-Ig, PTG), anti-DYKDDDDK tag Polyclonal (20543-1-AP, PTG), HT-DNA (D6898, Sigma), 2'3'-cGAMP (HY-100564A, MCE), diABZI (HY112921B, MCE), DMXAA (HY-10964, MCE), CCK-8 (KTA1020-1000T), Hoechst (Boston, USA), opti-MEM (2427634, Gibco), protease inhibitor (C0001, TargetMol).

The process of GUP extraction: The licorice would be washed, dried, sliced, and then licorice with distilled water was put in a vessel, heated, and kept 80 ℃ for 2 h and stirred regularly. The filtrates were combined and concentrated, extracted three times with ethyl acetate, recovered the aqueous layer and discarded the ethyl acetate. Subsequently, the water layer was placed in AB-8 macroporous adsorbent resin, washed with distilled water and recycled water parts. Finally, the recovered water part was dried in a freeze dryer for 48 h to access purified GUP (company supplied).

2.2 Animals

C57BL/6 mice (female, 8 weeks old) were purchased from SPF Biotechnology Co, Ltd (Beijing, China). All mice were housed in cages with unrestricted access to food and water under specific pathogen-free (SPF) conditions. The ambient humidity is 50 ± 5% and the temperature is 20-22°C. The Fifth Medical Center of the Chinese People's Liberation Army General Hospital approved all animal experimental procedures used in this study. These procedures were all carried out in accordance with guidelines for the care and use of laboratory animals.

2.3 The quality control of GUP

We used ion chromatography (ThermoFisher ICS5000) to determine the monosaccharide composition of GUP. The detector was electrochemical detector. The chromatographic column used is DionexCarbopac^{TM}PA20 (3*150). The chromatographic mobile phase: solvent A, H_2O and solvent B, 15 mM NaOH and solvent C, 15 mM NaOH and 100 mM NaOAC. The flow rate was 0.3 mL/min, the injection volume was 5 μL, and the column temperature was set at 30ºC. 16 monosaccharide standards including glucose, galactose and glucuronide were dissolved as controls. The 10mg sample was accurately weighed in the ampoule, 2 mL 3M TFA was added and hydrolyzed at 120°C for 3 h. The acid hydrolysis solution was accurately aspirated and transferred to a tube, which was blown dry with nitrogen. 5 mL of water was added and vortexed and mixed, 50 μL was aspirated and 950 μL of deionized water was added and centrifuged at 12000 rpm for 5 mins. Then, it is tested on an ion chromatograph.

2.4 Cell culture

Mouse bone marrow-derived macrophages (BMDMs) was isolated from bone marrow of 10-week-old female mice and incubated with DMEM medium containing 50 ng/mL murine macrophage colony-stimulating factor (MCE, Mon-mouth, NJ, USA) with the addition of 1% penicillin/streptomycin (Macgene) and 10% fetal bovine serum (Gibco, Rockford, IL, USA). Human primary monocytes (THP-1) were obtained from ATCC and cultured in RPMI 1640 medium. HEK-293T cells were provided by Tao Li (National Center of Biomedical Analysis). HEK-293T cells was cultured in DMEM medium. Healthy volunteers provided the human PBMCs, which

were cultivated in RPMI 1640 medium. All cell lines were developed at 37°C in an incubator with 5% CO_2 humidity.

2.5 Activation of the cGAS-STING Pathway

Human PBMCs , BMDMs cells and THP-1 cells (with PMA) were inoculated overnight at a density of 1.2×10^6, 1.3×10^6 and 2.5×10^6 cells/mL in well plates respectively. Next day, the GUP was dissolved in opti-MEM and filtered through a 0.22 μM non-pyrogenic filter membrane. After that, cells were treated with GUP for 1 h. Then, cells were transfected with HT-DNA (2.5 μg/mL) or 2'3'-cGAMP (2 μg/mL), or addition of diABZI (10 mmol/mL) or DMXAA (2.5 μg/mL). After 2 h, cell lysates were collected for western blotting. After 4 h, lysates from cells lysed with TRIZOL were collected for qPCR.

2.6 Cell viability assay

This experiment adopted the Cell Counting Kit 8 (CCK-8) to detect cell viability. THP-1 and BMDMs cells were seeded into 96-well plates and incubated overnight in a cell incubator. The cells were then treated with different concentrations of GUP for 12 h. CCK-8 reagent was added into the cell medium and incubated together with the cells in the incubator for 1 h. Finally, the optical density (O.D.) values at a wavelength of 450 nm were measured.

2.7 Enzyme-linked immunosorbent assay

Mouse serum and intraperitoneal lavage liquid were tested by mouse IL-6 (1210602, Dakewe), mouse TNF-α (1217202, Dakewe) and IFN-β (luex-mifnbv2, InvivoGen) according to the manufacturer's instructions.

2.8 Western blotting

The conventional method of protein extraction was used[31]. Cell supernatant was collected, and centrifuged for 5 mins, and whole cell lysate was prepared using 1× RIPA buffer. The supernatant of the centrifuged cell supernatant was then discarded and the fully lysed cell lysate was added. The protein samples were separated at 100 V by 10% SDS-PAGE and the gels were transferred onto PVDF membranes by a wet transfer system. The PVDF membrane was then closed at room temperature in 10% skimmed milk for 1 h, incubated with the primary antibody overnight at 4°C, and then incubated with the matching secondary antibody for 1 hour at room temperature. Washing with TBST was required between each step. Detection of protein bands used enhanced chemiluminescence reagents (Millipore, Massachusetts, USA).

2.9 Reverse Transcriptase Quantitative PCR

The TRIZOL reagent (Sigma, 93289) was used to extract total RNA in accordance with the manufacturer's instructions. cDNA was transcribed from equal amounts of RNA using the RT Master Mix (A230, GenStar). Real-time PCR was performed using the SYBR Green qPCR Master Mix (HY-K0501, MCE). Primer sequences are shown in Table 1, Table 2.

TABLE 1 Mouse primers for quantitative real-time PCR analysis

Target gene	Sequence (50–30)
mTNF-α-	GGGCAGTTAGGCATGGGAT
	TGAGCCTTTTAGGCTTCCCAG
mIFN-β	TCCGAGCAGAGATCTTCAGGAA

	TGCAAC CACCACTCATTCTGAG
mCXCL10	ATCATCCCTG CGAGCCTATCCT
	GACCTTTTTTGGCTAAACGCTTTC
mIL-6	CACTTCACAAGTCGGAGGCT
	CTGCAAGTGCATCATCGTTGT
mGAPDH	CGGAGTCAACGGATTTGGTC
	GACAAGCTTCCCGTTCTCAG
mActin	GGCTGTATTCCCCTCCATCG
	CCAGTTGGTAACAATGCCATGT

TABLE 2 Human primers for quantitative real-time PCR analysis

Target gene	Sequence (50–30)
hTNF-α-	CCTCTCTCTAATCAGCCCTCTG
	GAGGACCTGGGAGTAGATGAG
hIFN-β	TCCAAATTGCTCTCCTGTTG
	GCAGTATTCAAGCCTCCCAT
hCXCL10	TGGCATTCAAGGAGTACCTC
	TTGTAGCAATGATCTCAACACG
hIL-6	ACTCACCTCTTCAGAACGAATTG
	CCATCTTTGGAAGGTTCAGGTTG
hGAPDH	CGGAGTCAACGGATTTGGTC
	GACAAGCTTCCCGTTCTCAG
hActin	CATGTACGTTGCTATCCAGGC
	CTCCTTAATGTCACGCACGAT

2.10 STING oligomerization assay

Oligomerization experiments of STING was performed as previously reported[32].

Briefly, cell lysates in the native sample buffer were loaded to the native-PAGE gel and electrophoresed for 50 mins at 25 mA, and then analyzed by immunoblotting with anti-STING antibody.

2.11 Immunoprecipitation assay

For immunoprecipitation assay, HEK-293T cells were inoculated overnight and then transfected with Flag-IRF3 or Flag-TBKI and HA-STING or HA-IRF3 plasmids for 18 h. After the treatment with GUP for 4 h, cell pellets were collected and resuspended in lysis buffer (50 mM Tris [pH 7.45], 0.5% [v/v] Triton X-100, 150 mM NaCl, and 1% [m/v] containing protease inhibitors. In addition, the supernatant was incubated with M2 (A2220, Sigma) beads for 4 h at 4°C. The samples were washed three times with the lysis buffer, denaturalized by boiling with 1×RIPA loading buffer for 15 mins and analyzed by immunoblotting.

2.12 In vivo mice model of STING agonist (DMXAA) and CLP mice model

In order to assess the effect of GUP in vivo, we used C57BL/6 wild female mice (8 weeks old) and pre-administered GUP by gavage for 1 week, followed by intraperitoneal injection (i.p.) of DMXAA (25 mg/mL). After 4 h, mice were euthanized, serum and intraperitoneal lavage liquid were collected and DMXAA-induced cytokine levels were measured. GUP was dissolved in 0.9% saline and then administered to mice by gavage. DMXAA was dissolved in 1:1 with PEG800: normal saline.

CLP sepsis was induced with C57BL/6 female mice (8 weeks old) as described previously[14]. Briefly, the mice were anesthetized and the abdominal surgical area

was sterilized. A small midline abdominal incision was then made, and the cecum was exteriorized and ligated the cecum with silk. We punctured the ligated portion of the cecum with a 22-gauge needle twice before returning it to the abdominal cavity. The abdomen was eventually stitched up in two layers. For sham surgery, the cecum was exteriorized and then returned to the abdominal cavity without ligation or puncture. Immediately after the operation the mice were injected subcutaneously with 1 mL of Ringer's solution. After 72 h, the mice were executed through cervical dislocation and lung, heart, liver and kidney tissues were taken. One of the lung tissues was paraffin-embedded, followed by hematoxylin-eosin (H&E) staining using standard techniques. Heart, liver and kidney tissues were homogenized and then RNA was extracted for qPCR.

2.13 Statistical analysis

All experimental data were analyzed using the software GraphPad Prism 8 and SPSS statistics 26.0. The statistical evaluation was conducted by an unpaired Student's t-test between two groups or one-way ANOVA with Dunnett's post hoc test was used for multi groups. The results were shown as Mean ± Standard error of mean (SEM), and P values were presented by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Result

3.1 The quality control of GUP and cytotoxic effects of GUP

In order to clarify the evidence for the chemical characterization of GUP, the composition of the monosaccharides was determined by ion chromatography. The monosaccharide composition of GUP was measured by comparison with the retention

times and chromatograph peaks of standard monosaccharides including glucose, galactose and glucuronide acid. Data revealed that the molar ratio of glucose, glucuronide acid and galactose in the GUP sample was 0.902, 0.056, 0.043 respectively (Figure S1a, b). We examined the cytotoxicity of GUP in BMDMs and THP-1 cells to learn more about its impact on the activation of the cGAS-STING pathway. THP-1 and BMDMs cells were treated with different concentrations of GUP for 12 h. The cell viability assay showed that GUP had no cytotoxicity at concentrations below 3 mg/mL in both cells (Figure S1 c, d). Subsequent experiments were carried out at non-toxic doses.

3.2 GUP inhibits the activation of HT-DNA-induced cGAS-STING pathway

Next, we assessed the effect of GUP on herring testis DNA (HT-DNA)-induced activation of the cGAS-STING signaling pathway. HT-DNA enters the cytoplasm through transfection and can be sensed by cGAS and then activate STING[33]. Monocytes and macrophages, as secretory cells, are essential for the modulation of the immune response, as well as the development of inflammation[34]. So, we used BMDMs and THP-1 cells to explore whether GUP affected the cGAS-STING pathway. Phosphorylation of IRF3 is a key downstream signaling event in the cGAS-STING pathway. Using immunoblotting analysis to detect related proteins, we found that GUP treatment suppressed the phosphorylation of IRF3 induced by HT-DNA (Figures 1a, 1b). Meanwhile, we assessed the effect of GUP on mRNA expression of downstream genes related to the cGAS-STING pathway, for example IFN-β, IL-6 and TNF-α. Surprisingly, GUP inhibited the mRNA expression of these genes in BMDMs

(Figures 1c, 1d and 1e) and THP-1 (Figures 1f, 1g and 1h) cells. More importantly, GUP also inhibited HT-DNA-induced mRNA expression of IFN-β, IL-6 and TNF-α in human PBMCs (Figures 1i, 1j and 1k). In conclusion, these data demonstrate that GUP can suppress the activation of HT-DNA-induced cGAS-STING pathway in vitro.

3.3 GUP inhibits multiple stimulus-induced activation of the cGAS-STING pathway

We next investigated GUP affects the activation of the cGAS-STING pathway induced by multiple STING agonists to determine whether it has a broad-spectrum inhibitory effect on the cGAS-STING pathway. Some natural or enzymatically synthesized STING agonists, such as 2′3′-cGAMP, 2′2′-cGAMP, c-di-AMP, diABZI, c-di-GMP, c-di-IMP and DMXAA, can also induce the expression of type I IFNs and other pro-inflammatory factors[35-39]. We further study found that GUP inhibited the phosphorylation of IRF3 induced by various typical STING stimulators (HT-DNA, 2'3'-cGAMP, diABZI, DMXAA) in BMDMs (Figure 2a) and THP-1 (Figure 2f) cells. In addition, to further validate the above results, we also performed qPCR on its related downstream genes. Interestingly, GUP significantly inhibited the mRNA expression of IFN-β, IL-6, TNF-α and interferon-stimulated genes (CXCL10) activated by multiple STING stimulators in BMDMs (Figures 2b, 2c, 2d and 2e) and THP-1 (Figures 2g, 2h, 2i and 2j) cells. All in all, these results strongly demonstate that GUP has an important regulatory role in the activation of the cGAS-STING pathway triggered by multiple stimulus.

3.4 GUP disrupts nuclear translocation of IRF3

Nuclear translocation is an important function in transcription. IRF3 is phosphorylated to form a dimer that translocates to the nucleus and thereby regulates the expression of target genes, inducing the expression of type I IFNs[40-42]. Here, we explored whether GUP could inhibit the nuclear translocation of IRF3, thereby suppressing transcriptional activity. Therefore, we examined the expression of IRF3 in the nucleus and cytoplasm of THP-1 cells. As shown in the picture (Figure 3a), GUP inhibited the nuclear accumulation of IRF induced by 2'3'-cGAMP stimulation. To verify these results, we performed an immunofluorescence assay. Our results showed (Figure 3b), GUP significantly reduces diABZI induced nuclear aggregation of IRF3. The present study demonstrated that GUP blocked IRF3 transcriptional activity and thereby suppressed the expression of type I IFNs.

3.5 GUP inhibits the activation of cGAS-STING pathway by suppressing STING-TBK1 and TBK1-IRF3 interactions

Next, we aim to explore the mechanisms of GUP affects the activation of the cGAS-STING pathway. Our previous research demonstrated that GUP could suppress the phosphorylation of IRF3 and nuclear translocation of IRF3, a key downstream signaling event in the cGAS-STING pathway. Therefore, we will further explore the effect of GUP on the upstream of the cGAS-STING pathway. Studies have shown that cGAMP induces a conformational change in STING leading to oligomerization of STING and subsequently recruits TBK1 to activate downstream pathways[40]. However, our study showed that GUP did not inhibit 2'3'-CGAMP-induced STING oligomerization (Figure 3c). STING is a scaffold protein that, upon binding to CDNs,

recruits downstream signaling proteins[32]. After STING recruits TBK1, IRF-3 transcription factors are subsequently recruited to the signaling complex and phosphorylated by TBK1, which in turn regulates downstream signaling pathways[41, 43, 44]. Thus, binding between STIING, TBK1 and IRF3 is essential for the activation of the cGAS-STING pathway. To this end, we examined whether GUP inhibited the interaction between three key proteins in the cGAS-STING pathway, STING, TBK1 and IRF3. Notably, our results showed that in exogenous scenarios, GUP inhibited STING interactions with TBK1 (Figure 3d) and TBK1 interactions with IRF3 (Figure 3e), but not STING-IRF3 interactions (Figure 3f). In conclusion, these results suggest that GUP inhibits the activation of the cGAS-STING pathway by inhibiting the interaction of STING with TBK1 and TBK1 with IRF3.

3.6 GUP inhibits the activation of cGAS-STING in vivo

We next investigated the effect of GUP on the activation of the cGAS-STING pathway in vivo. DMXAA, a murine agonist of the interferon gene (STING) stimulator[45, 46], was also a potent inducer of type I IFNs and inflammatory cytokines, so we chose DMXAA for our in vivo study. Mice were given GUP (400 mg/kg/day) pretreatment for 1 week, followed by intraperitoneal injection of DMXAA. After 4 h, intraperitoneal lavage liquid and serum were collected from the mice and the levels of IFN-β, IL-6 and TNF-α in the intraperitoneal lavage fluid and serum were measured by Elisa kits. Our results showed that DMXAA stimulation increased the levels of IFN-β, IL-6 and TNF-α in the intraperitoneal lavage liquid (Figures 4a, 4b and 4c) and serum (Figures 4d, 4e and 4f) of mice. Notably, GUP

significantly reduced DMXAA-mediated expression of IFN-β, IL-6 and TNF-α. Thus, GUP can inhibit the activation of the cGAS-STING pathway in vivo.

3.7 GUP alleviates CLP through regulating cGAS-STING signaling pathway

Study showed significant activation of cGAS-STING pathway in CLP-induced sepsis[13]. In recent years, various models of sepsis have been studied, and CLP model is the most associated animal model for clinical sepsis[47, 48]. To determine the effect of GUP on sepsis, CLP was performed as described previously[14]. GUP (400 mg/kg/day) was pretreated to mice for 1 week and repeated oral administration to mice at 12, 24, 48 and 72 h after CLP. Through the analysis of H&E staining of lung tissue of mice, we found that CLP-induced lungs increased leukocyte infiltration and significant accumulation of inflammatory cells in the alveolar space, consistent with previous studies[14]. Our results showed that GUP reduces damage to lung tissue (Figure 5a). Furthermore, in CLP-induced heart, liver and kidney tissues, the mRNA expression of IFN-β and TNF-α was increased at 72 h, whereas GUP decreased the mRNA expression of IFN-β (Figures 5b, 5c and 5d) and TNF-α (Figure 5e, 5f and 5g). In general, these data suggest a protective effect of GUP against CLP sepsis. Combining the results of previous experiments and the role of the cGAS-STING pathway in the inflammatory response[6], we speculate that GUP may exert its anti-inflammatory effects by down-regulating the cGAS-STING pathway.

Discussion

Recently, the cGAS-STING pathway has been increasingly studied. As the study progressed, researchers discovered that the cGAS-STING pathway was significantly

expressed in a number of inflammatory diseases, such as sepsis[13], kidney injury[49] and lung injury[50]. Sepsis is an acute disease caused by an infection or trauma resulting in a systemic inflammatory response caused by microbial invasion, resulting in a dysregulated host response leading to organ dysfunction and a serious risk to human life and health[51]. Therefore, if inhibitors of the cGAS-STING pathway can be identified, there is hope for targeted therapy for sepsis caused by the cGAS-STING pathway. Licorice is a traditional medicinal plant that has demonstrated antiviral and anti-inflammatory activity[17, 52, 53]. This article reports for the first time that GUP, the main active component of licorice, suppressed the activation of cGAS-STING both in vitro and in vivo, revealing the role of GUP in regulating cGAS-STING-mediated sepsis.

It has been previously reported that GUP have antioxidation, antibacterial and anti-inflammatory activities, but little research has been done on anti-inflammatory activities[28, 54]. Zhao et al. found that GUP alleviated the symptoms of DSS-induced UC mice by suppressing the expression of IL-1, IL-6 and TNF-α in serum [30]. Although its anti-inflammatory effects have been studied to some extent, its exact mechanism of action and targets are not yet clear. Our research showed that GUP inhibited the phosphorylation of IRF3 and the expression of mRNA of related downstream gene to the cGAS-STING signaling pathway, such as IFN-β, IL-6, TNF-α and CXCL10 induced by various canonical STING stimulators (HT-DNA, 2'3'-cGAMP, diABZI and DMXAA). This suggests that GUP does play a role in the activation of the cGAS-STING signaling pathway and is a broad-spectrum inhibitor of

the cGAS-STING pathway. Previous research has revealed that the STING agonist DMXAA causes systemic inflammatory responses and shock-like symptoms in mice[45, 55]. It is noteworthy that GUP pretreatment significantly reduced the expression of systemic cytokine in DMXAA-induced mice, such as IFN-β, IL-6 and TNF-α. This result suggests that GUP can suppress the activation of the cGAS-STING pathway in vivo.

STING is activated by cGAMP and recruits TBK1 and IRF3. IRF3 forms a complex with STING and TBK1 and is phosphorylated by TBK1, thereby regulating downstream gene expression[56, 57]. Increasing data demonstrate that the interaction between three proteins, STING, TBK1 and IRF3 are important for the activation of the cGAS-STING pathway[32, 43, 56]. Our results show that GUP can inhibit STING-TBK1 and TBK1-IRF3 interactions, which explains why GUP can inhibit the activation of the cGAS-STING pathway.

Our ultimate aim is to assess the therapeutic potential of GUP for CLP sepsis. Walker's group demonstrated for the first time that STING knockout (KO) mice were protected from CLP induced sepsis[58]. Li's laboratory further demonstrated increased cGAS-STING pathway in human sepsis and found that blocking cGAS-STING pathway protects against sepsis-associated acute liver injury[13]. These data confirm the importance of the cGAS-STING pathway in the development of sepsis. The release of inflammatory cytokines often leads to sepsis, and suppression of inflammation is a strategy for treating sepsis[59]. Previous studies have shown that type I IFNs is an essential cytokine that promotes and regulates immune and

inflammatory responses, and its inappropriate expression leads to the death of various cells, which is essential for the septicemic response[13, 60, 61]. TNF-α is a key immune system regulator whose overexpression may play a role in the development of inflammatory disorders[62]. Our results showed that CLP mice showed dysfunction of the heart, liver and kidney, such as increased release of IFN-β and TNF-α, which was consistent with previous findings[14]. H&E staining of the lung tissue also showed some degree of damage to the lung tissue. Surprisingly, the GUP treatment improved these symptoms. In view of the above results, we hypothesize that the ameliorative effect of GUP on CLP sepsis may act by mediating immune-related pathways, especially the cGAS-STING pathway. However, whether GUP has a protective effect against other cGAS-STING-mediated inflammatory diseases needs to be further explored.

In conclusion, our study identifies a potent inhibitor of the cGAS-STING pathway. GUP inhibited the activation of the cGAS-STING pathway in vitro and in vivo, reducing the expression of related downstream gene. Mechanistically, GUP inhibited the cGAS-STING pathway by inhibiting the interaction of STING with TBK1 and TBK1 with IRF3. At the same time, it also provides a potential therapeutic agent for cGAS-STING pathway-induced related inflammatory diseases, particularly sepsis.

Data availability

All data will be made available upon request.

Author contributions

ZFB, SLQ, GX and XHX propose research options and obtain funding for research. SWH, WK, SLQ and PH designed the study protocol and completed most of the experiments. JZ, JB, HL and WQM provide assistance with the investigation and analyzed the data. JCW, MTH and ZYW help with animal experiments. SWH drafted first draft of paper, ZFB, XG, XHX revised paper. SLQ is a mentor of SWH.

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Declarations

Conflict of interest The authors declare that we have no financial and personal relationships with other people or organizations

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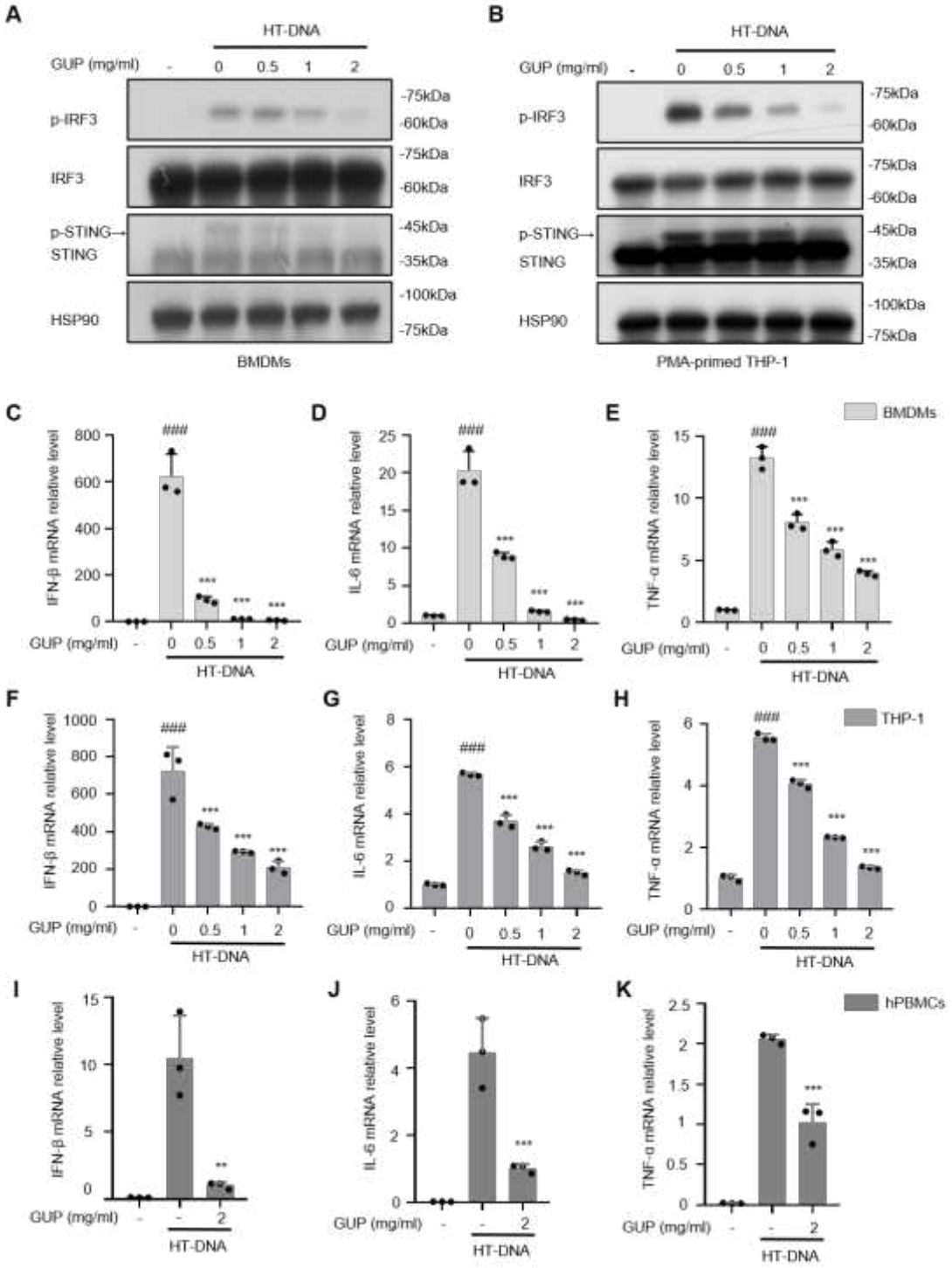
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Figure Legends



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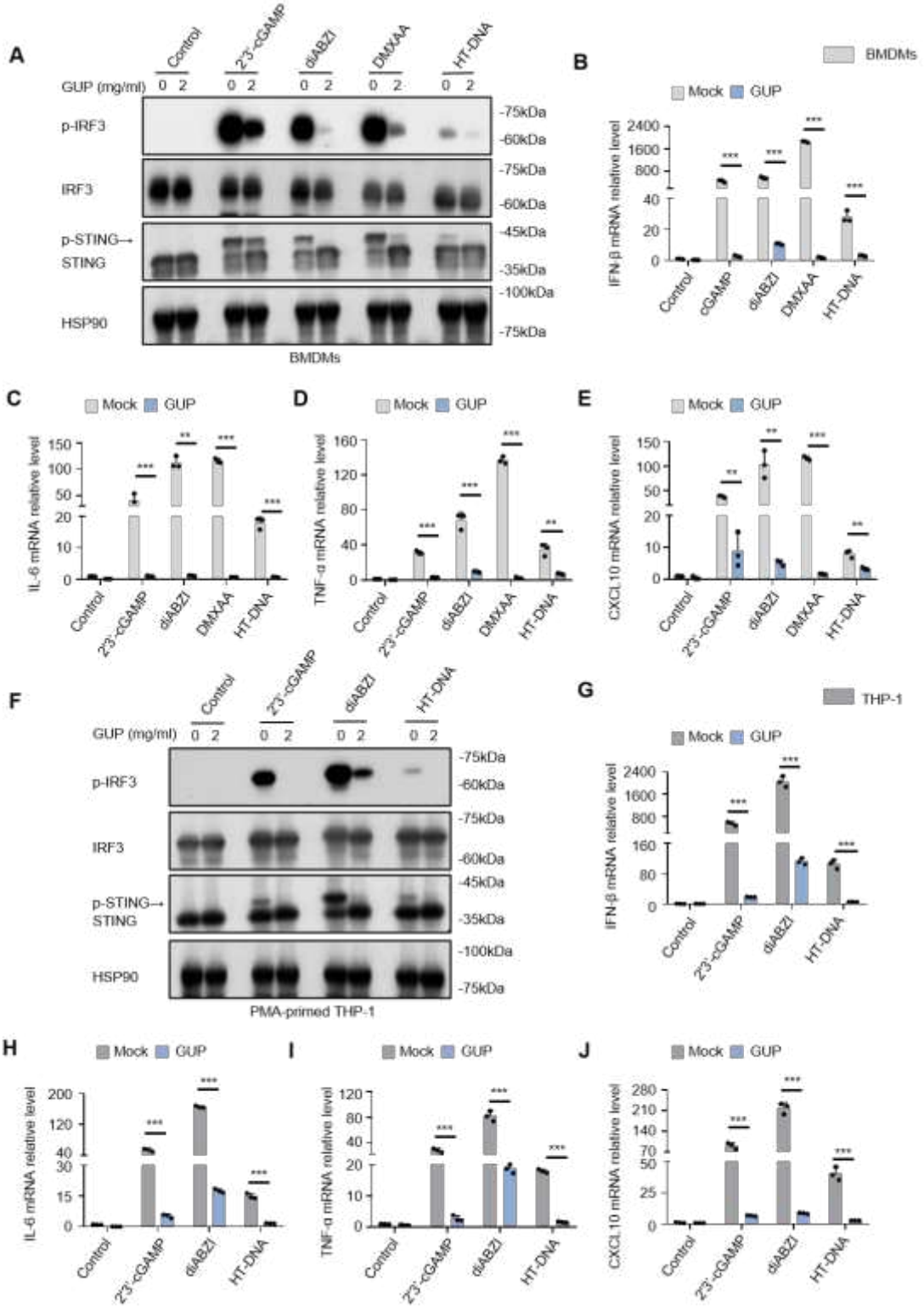
628 FIGURE 1 GUP inhibits the activation of HT-DNA-induced cGAS-STING pathway.

629 (a) BMDMs and (b) THP-1 cells were pretreated with the different concentrations of

630 GUP for 1 h, transfected with HT-DNA for 2 h. The cell lysates were subjected to

631 Western blotting to determine phosphorylated p-IRF3, IRF3, p-STING and STING

protein levels. HSP90 was used as a loading control. The mRNA expression of IFN-β, TNF-α and IL-6 was measured by quantitative PCR assay in (c, d, e) BMDMs cells and (f, g, h) THP-1 cells. The experimental steps were followed as above (c, d) except transfected with HT-DNA for 4 h. (i, j, k) Human PBMCs were treated with GUP for 1 h and then transfected with HT-DNA for 4 h. The mRNA expression of IFN-β, TNF-α and IL-6 is measured in qPCR. The ΔΔCt method was used to quantify relative changes. One-way ANOVA statistics were used to compare the groups. The results were shown as mean ± SEM (n=3). $^{###}p < 0.001$ vs. the control group. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. the model group.



641

642 FIGURE 2 GUP inhibits multiple agonist-induced activation of the cGAS-STING

643 pathway.

644 (a) BMDMs and (f)THP-1 cells were pretreated with GUP (2 mg/ml) for 1h,

transfected with 2'3'-cGAMP and HT-DNA, and addition of diABZI and DMXAA for 2 h. Phosphorylated expression of p-IRF3, IRF3, p-STING and STING was determined by Western blotting. HSP90 was used as a loading control. The mRNA expression of IFN-β, IL-6, TNF-α and CXCL10 was measured by quantitative PCR assay in (b, c, d, e) BMDMs cells and (g, h, i, j) THP-1 cells. The experimental steps were followed as above (a, f) except transfected 2'3'-cGAMP and HT-DNA, and addition of diABZI and DMXAA for 4 h. Unpaired two-tailed Student's t tests statistics were used to compare the groups (n=3). The results were shown as mean ± SEM, and p value were presented by *p < 0.05, **p < 0.01 and ***p < 0.001 mock group (no administration) versus group (administration).

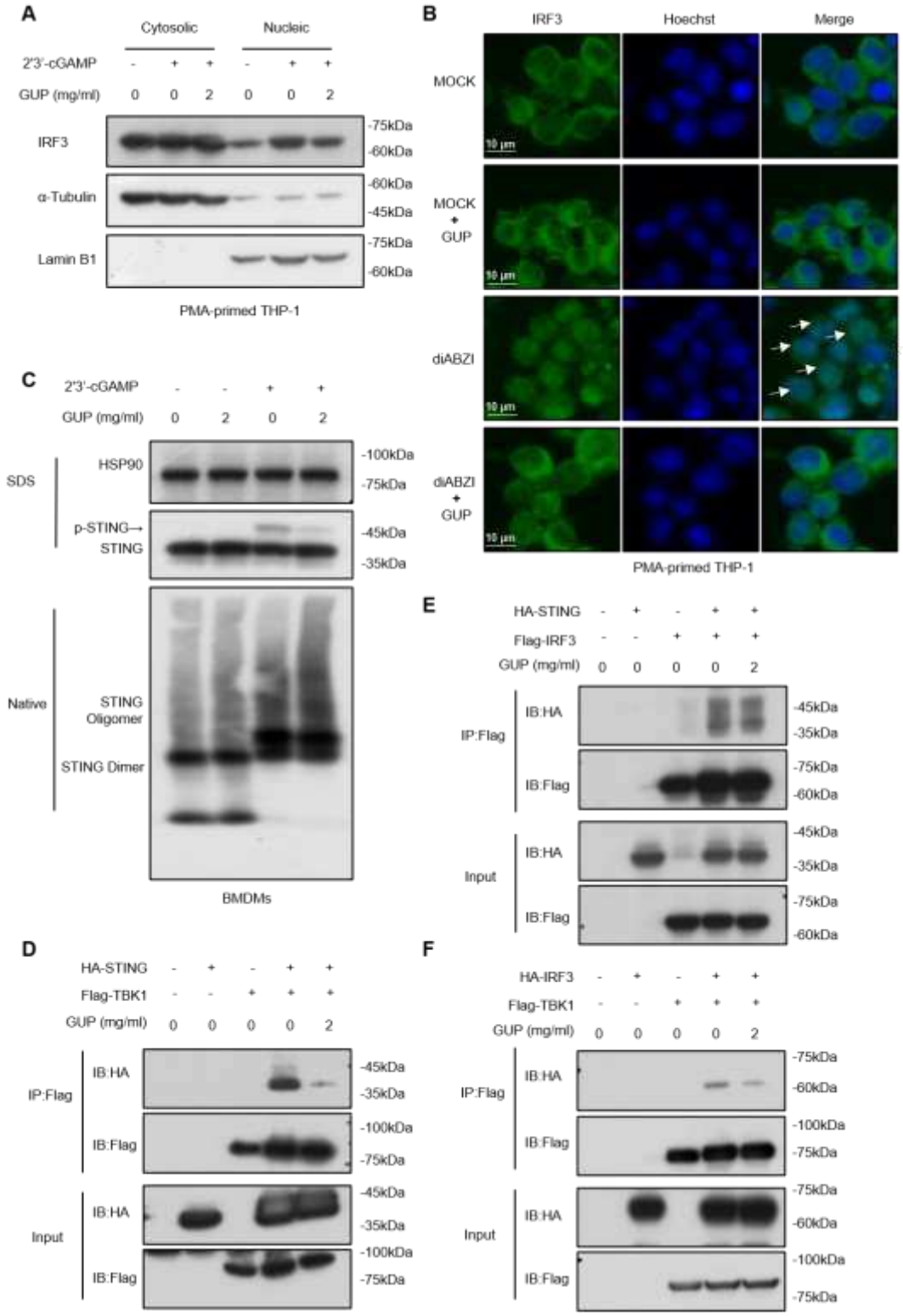
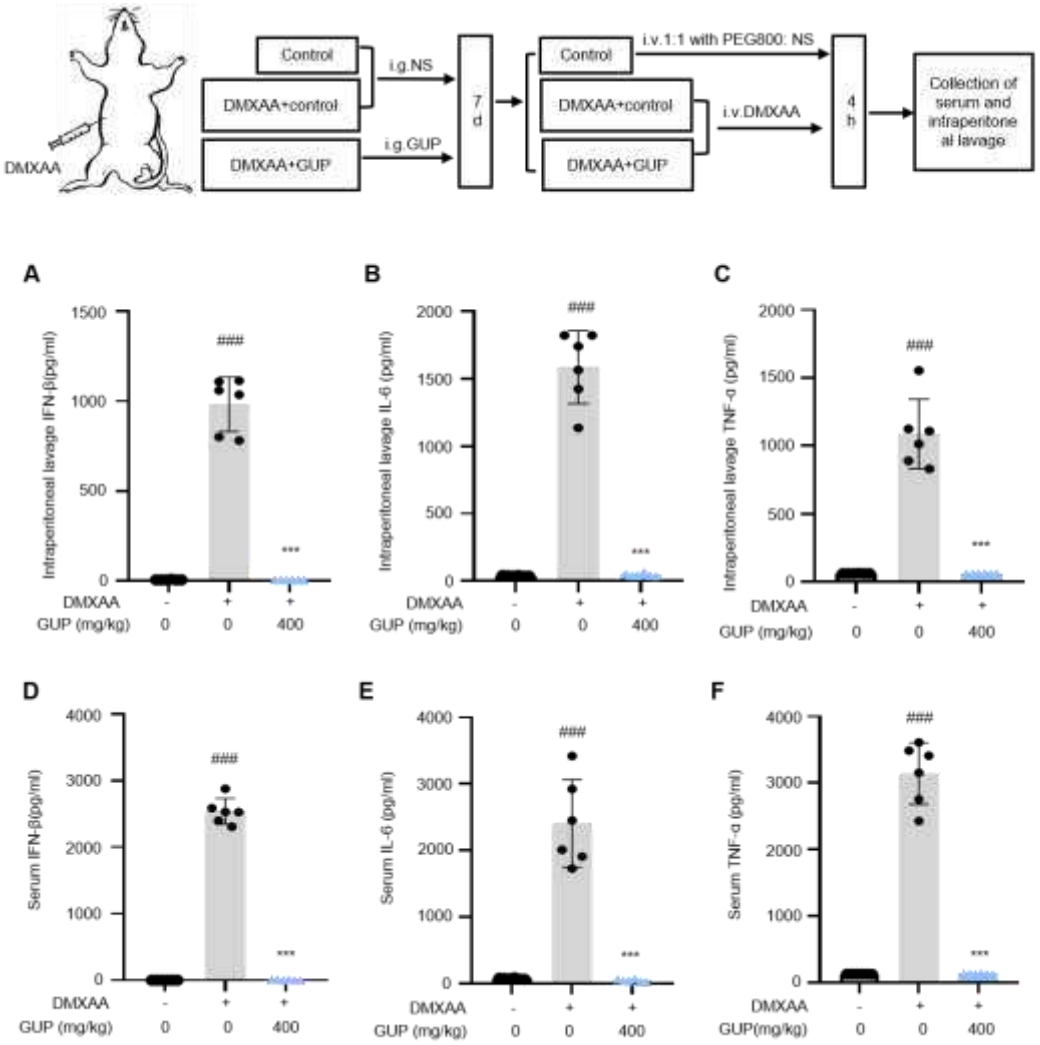


FIGURE 3 GUP disrupts nuclear translocation of IRF3 and inhibits STING-TBK1 and TBK1-IRF3 interactions.

(a) THP-1 cells were treated with GUP for 1 h, then transfected with 2‘3’-cGAMP for

2 h before lysing. Protein expression in cytosolic and nuclear lysate of THP-1 cells treated with GUP were analyzed by Western blot to determine the cellular distribution of IRF3. (b) THP-1cells were treated with GUP for 1h, then stimulated with diABZI for 2 h and stained for IRF3. The blue Hoechst (nucleus) image is superimposed on the green IRF3 image. At 20 original magnification, pictures were shot for each circumstance. Scale bars represent 10 μm. (c) BMDMs cells were pretreated with GUP for 1 h and then transfected with 2‘3’-cGAMP for 1 h. Collection of lysates for immunoblotting to detect phosphorylation and oligomerization of STING. (d) Flag-TBK1 or (e) Flag-IRF3 plasmids was co-transfected with HA-STING plasmids into HEK-293T cells. (f) Flag-TBK1 plasmids was co-transfected with HA-IRF3 plasmids into HEK-293T cells. 18 h after transfection, cells were treated with GUP for 4 h. Immunoprecipitation was performed with anti-DYKDDDDK (Flag) affinity gel agarose beads. Western blot analysis has been shown.



672

673 FIGURE 4 GUP inhibits the activation of cGAS-STING in vivo.

674 Mice were pre-treated with GUP for 1 week, then i.p. injected with DMXAA (25

675 mg/kg) and treated for 4 h. The levels of IFN-β, IL-6 and TNF-α respectively in the (a,

676 b, c) intraperitoneal lavage and (d, e, f) serum were measured by ELISA. The data

677 were displayed by Mean ± SEM from three biological replicates. One-way ANOVA

678 statistics were used to compare the groups. $^{###}p < 0.001$ means compared to the

679 control group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus the DMXAA group. ($n = 6$

680 mice per group).

681

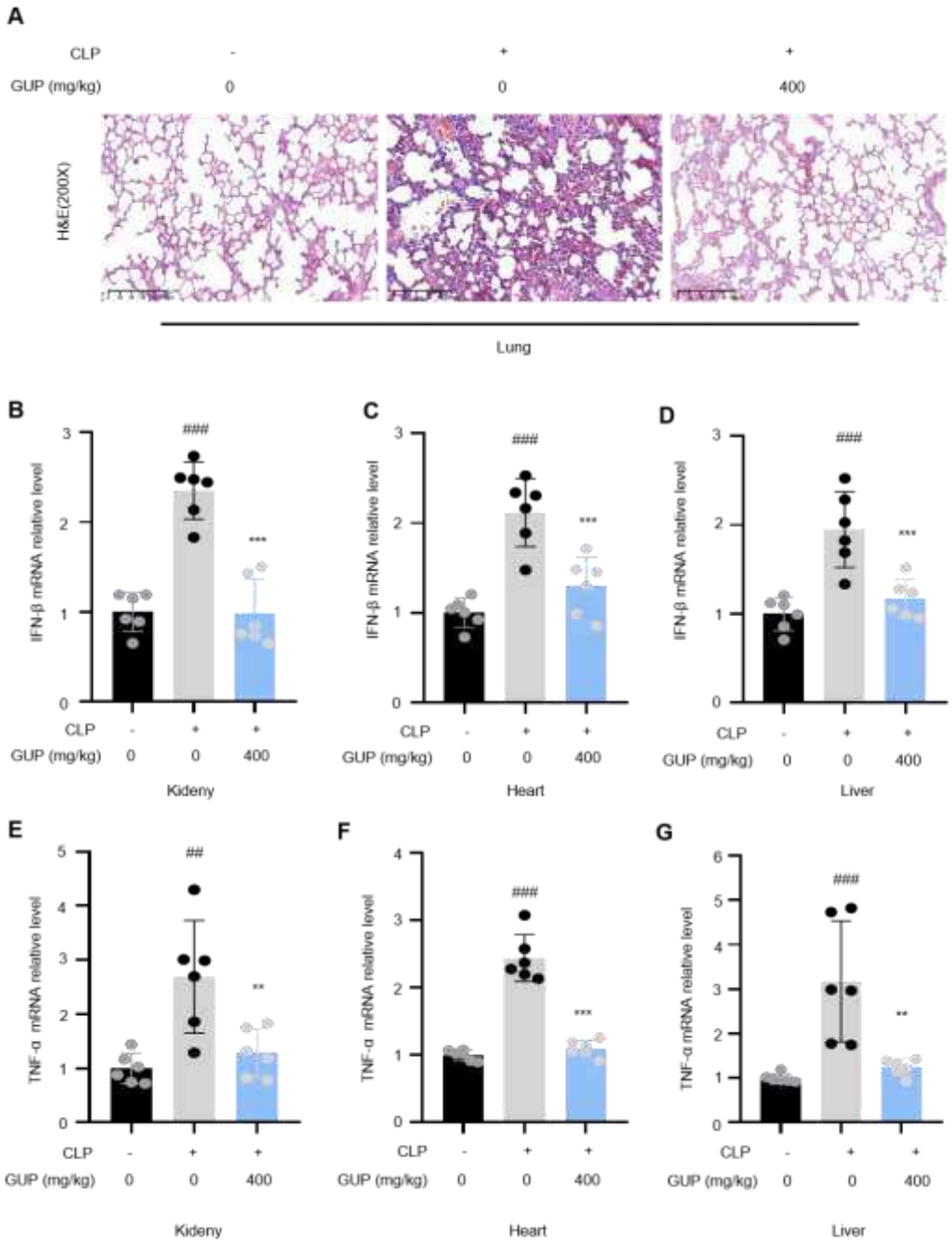
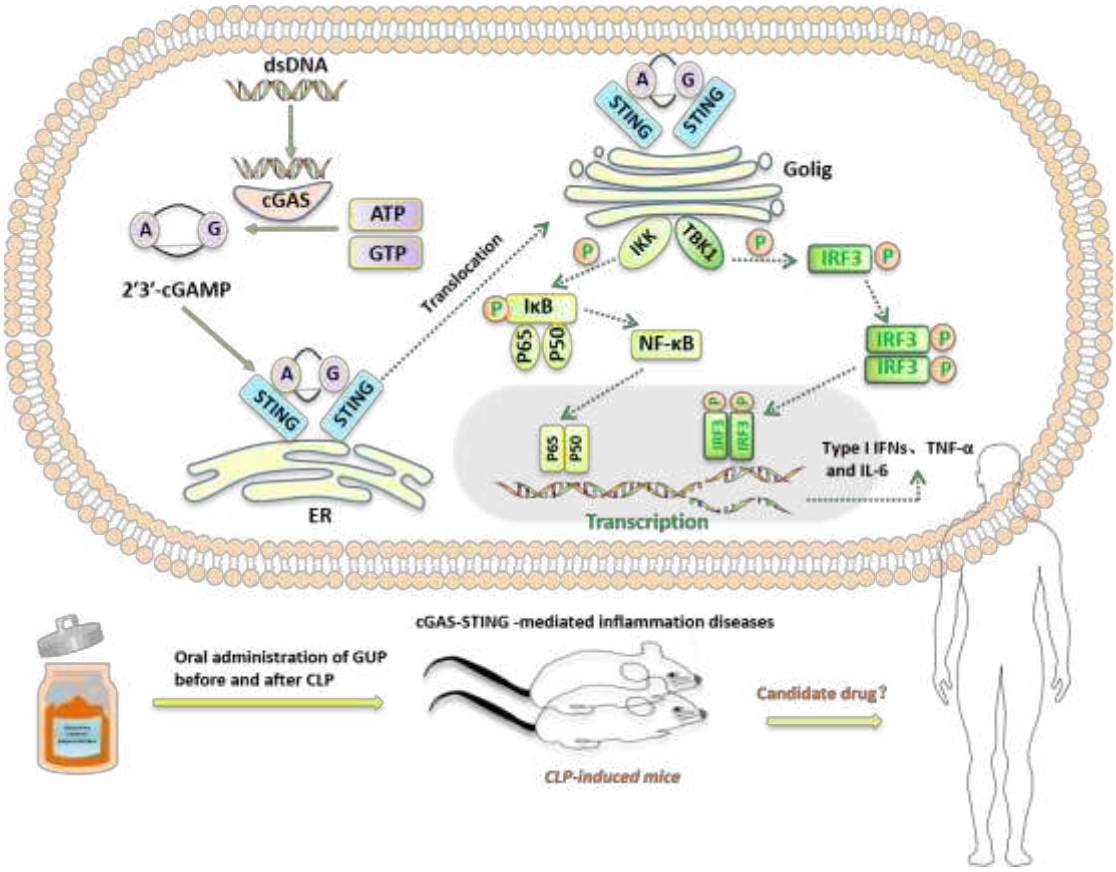


FIGURE 5 GUP alleviates CLP through regulating cGAS-STING signaling pathway. (a) Representative H&E staining of lung tissues. Scale bar: 100 μm. (n = 6 mice per group). (b, c, d) IFN-β and (e, f, g) TNF-α cytokine mRNA in Kideny, Heart and Liver. One-way ANOVA statistics were used to compare the groups. The results were shown as Mean ± SEM. $^{###}p < 0.001$ means compared to the control group; *p < 0.05, **p < 0.01 and ***p < 0.001 versus the CLP group. (n = 6 mice per group).


dsDNA
cGAS
ATP
GTP
A
G
2'3'-cGAMP
Translocation
STING
STING
ER
Golig
IKK
TBK1
IRF3
IκB
P65
P50
NF-κB
Transcription
Type I IFNs、TNF-α
and IL-6
cGAS-STING -mediated inflammation diseases
Oral administration of GUP
before and after CLP
CLP-induced mice
Candidate drug?

689