Am J Transl Res 2021;13(5):4080-4091
www.ajtr.org /ISSN:1943-8141/AJTR0131092

Original Article

CRISPLD2 attenuates pro-inflammatory cytokines production in HMGB1-stimulated monocytes and septic mice

Sheng Zhang[1*], Lei Pei[2*], Jinlong Qu[2*], Lizhu Sun[2], Weiwei Jiang[2], Wenfang Li[2], Zhaofen Lin[2], Dechang Chen[1]

[1]Department of Critical Care Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Department of Critical Care Medicine, Changzheng Hospital, Second Military Medical University, Shanghai, China. [*]Equal contributors.

Received January 30, 2021; Accepted March 4, 2021; Epub May 15, 2021; Published May 30, 2021

Abstract: HMGB1 has been identified as a pro-inflammatory mediator which leads to sepsis lethality. Previous studies suggested that CRISPLD2 had anti-inflammatory property and might severe as a therapeutic agent in sepsis. In the present study, we first conducted bioinformatic analysis to explore the expression profile of HMGB1 in septic survivors and non-survivors. We found that the serum HMGB1 level of septic non-survivors was significantly higher than that of septic survivors, and there was a positive correlation between CRISPLD2 and HMGB1 in mRNA expression in most of the cancer and normal tissue types, revealing a co-expression or dependency relationship between the two genes. In vitro, using cultured THP-1 cells, we confirmed that HMGB1 can induce the expression of CRISPLD2 in a time dependent manner through TLR4-dependent pathway. Given that CRISPLD2 and HMGB1 shared a wide range of time scales in gene expression and the anti-inflammatory property of CRISPLD2, we further verified that HMGB1 induced cytokines production might be partially reversed by CRISPLD2. In vivo, intravenously treatment of CRISPLD2 failed to rescue septic mice, although the serum levels of inflammatory cytokines were decreased. In conclusion, our study demonstrated that HMGB1 can act as stimuli to up-regulate the expression of CRISPLD2 in THP-1 cells, and in turn, increased CRISPLD2 can curtail HMGB1 induced pro-inflammatory cytokines production. Unfortunately, the anti-inflammatory effects of CRISPLD2 did not translate into survival benefit in mice with sepsis.

Keywords: Sepsis, HMGB1, CRISPLD2, inflammatory response, bioinformatics analysis

Introduction

Sepsis, a life-threatening syndrome caused by infection, is the leading cause of death in the intensive care units. Without timely treatment, sepsis can rapidly deteriorate into septic shock, multiple organ failure, and even death [1]. The pathogenesis of sepsis is rather complex. Usually, sepsis involves an uncontrolled inflammatory response triggered by the invasion of pathogens and their toxins, which is characterized by an overwhelming burst of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, interferon-γ, and macrophage migration inhibitory factor [2]. Besides, a highly conserved DNA-binding protein known as high mobility group box 1 (HMGB1), also acts as a potent inflammatory mediator and contributes to lethality in sepsis [3].

HMGB1 is a nuclear protein involved in various transcriptional processes. Under inflammatory conditions, it can be secreted from activated monocytes/macrophages and released from necrotic cells [4]. When HMGB1 is released into the extracellular environment, it can bind to related cell signaling transduction receptors, such as the T-cell immunoglobulin domain and mucin domain-3 (TIM3), receptor for advanced glycation end product (RAGE), Toll-like receptor (TLR)2, TLR4 and TLR9 and eventually stimulates the immune system [5]. In particular, The interaction between HMGB1 and TLR4 can activate nuclear factor nuclear factor κB (NF-κB) and up-regulate the expression of cytokines

such as IL-6 and TNF-α, which in turn promotes the release of HMGB1 and positively amplifies the effect of HMGB1 [6].

In previous studies, Wang et al. reported a novel LPS-binding protein, Cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2). The protein can directly bind to LPS in bloodstream and competitively inhibits LPS binding to its original target receptor-TLR4, consequently reduces LPS derived inflammation. Intriguingly, LPS itself can sever as a stimulus to induce the secretion and release of CRISPLD2 through TLR4 mediated signaling in vitro and in vivo. An even more interesting phenomenon is that a type of TLR4 specific antibody (anti-hTLR4-IgAmA), which was originally used for blocking LPS induced inflammation, could enhance secretion of CRISPLD2 from immunological cells [7].

Given that the high degree of similarity between LPS and HMGB1 in evoking inflammation, and that a certain protein (anti-hTLR4-IgAmA) is capable of interacting with TLR4 and induce the secretion of CRISPLD2, we suppose not only LPS, but also HMGB1 may act as a stimulus to up-regulate expression of CRISPLD2 in immunological cells. In addition, with regard to the finding that secreted CRISPLD2 can engage in neutralizing LPS thus curtails excessive inflammatory response raised by LPS stimulation. We will explore whether CRISPLD2 could also attenuate HMGB1 mediated inflammatory response in sepsis model.

Materials and methods

Bioinformatics analysis

In this section, we performed two series of bioinformatics analyses. First, we analyzed the relationship between HMGB1 expression and patient prognosis using microarray datasets downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Second, we explored the correlation between HMGB1 and CRISPLD2 across tissue types using data from The Cancer Genome Atlas (TCGA) database and the Genotype-Tissue Expression (GTEx) database.

Microarray data information: Using "sepsis" or "septic shock" as the keywords, with restriction the organisms to "homo sapiens", we searched the GEO database to identify Gene Series Expression (GSE) datasets that contain gene expression profiles and patient prognosis. Two eligible datasets (GSE26440 and GSE95233) were retrieved. Both of the datasets contain clinical information and corresponding gene expression profiles of whole blood samples from septic patients and healthy volunteers. For all the patients, only the samples collected at hospital admission were used for bioinformatics analysis.

Data processing, DEGs identification, and results visualization: Data normalization and gene differential expression analysis were performed using the limma package which was downloaded from the Bioconductor platform (http://www.bioconductor.org/packages/release/bioc/html/limma.html). Differentially expressed genes (DEGs) between septic survivors and non-survivors were identified using the criteria that $|logFC| > 0.1$ and Bonferroni-adjusted $P < 0.05$. DEGs were visualized using volcano plot. The correlation of mRNA expression between HMGB1 and CRISPLD2 in 31 normal tissue types and 33 cancer types were presented by dot plot, using $-\log_{10}P$ as horizontal coordinates and correlation coefficient (r) as vertical coordinates.

In vitro and in vivo experiments

Reagents: Escherichia coli LPS (O55:B5) was purchased from Sigma-Aldrich Co. LLC. and dissolved in PBS at required concentrations. Recombinant HMGB1 were purchased from Sino Biological Inc. and recombinant CRISPLD2 from Shanghai Southern Gene Technology co, Ltd. The antibodies targeting β-actin and CRISPLD2 were purchased from Sigma-Aldrich Co. LLC. and Novus Biologicals Inc., respectively.

Mice: 6-8-week-old Balb/c male mice (weight 20-25 g) were purchased from SIPPR-BK Experimental Animal Ltd. (Shanghai, China). All mice were bred in standard Specific Pathogen Free (SPF) conditions at the animal care facility of the Second Military Medical University under conditions at 21-24°C, 40-60% humidity, with lightening (12-h light/12-h dark cycle) and free access to food and water.

Animal experimental design: To determine the effect of CRISPLD2 on serum inflammatory factors levels, septic mice were injected with

Table 1. The primer sequence of RTqPCR

primers for TNF-α (Sense)	5'-CGAGTGACAAGCCTGTAGC-3'
primers for TNF-α (Antisense)	5'-GGTGTGGGTGAGGAGCACAT-3'
primers for IL-6 (Sense)	5'-GTAGCCGCCCCACACAGA-3'
primers for IL-6 (Antisense)	5'-CATGTCTCCTTTCTCAGGGCTG-3'
primers for IL-8 (Sense)	5'-AACTTCTCCACAACCCTCTG-3'
primers for IL-8 (Antisense)	5'-TTGGCAGCCTTCCTGATTTC-3'
primers for CRISPLD2 (Sense)	5'-CGCAGAATCCTGCTCCTTAG-3'
primers for CRISPLD2 (Antisense)	5'-GCCACTAGATCACCCTGGTC-3'
primers for β-actin (Sense)	5'-TGTGTTGGCGTACAGGTCTTTG-3'
primers for β-actin (Antisense)	5'-GGGAAATCGTGCGTGACATTAAG-3'

recombinant CRISPLD2 or equivalent volume of saline 6 hours post cecal ligation and puncture (CLP) surgery. Blood samples were obtained at 24 h after surgery for cytokines measurement. In addition, the survival time for septic mice injected with and without recombinant CRISPLD2 was documented.

CLP model: Sepsis was established using Cecal Ligation and Puncture (CLP) model as described previously [8]. In brief, laparotomy was performed on pentobarbital anaesthetized mice, and then the cecum was exteriorized, after which approximately half of the cecum was ligated with a silk suture and punctured with a 22-gauge needle to extrude a small drop of feces. Then the cecum was placed back into the peritoneal cavity following by the abdominal closure. Sham-operated control mice only received laparotomy without cecal puncture or ligation. All procedures abided by the guidelines of the Ethics Committee on Animal Experiments of the Second Military Medical University.

Cell culture and cellular experimental design: The human monocytic cell line THP-1 obtained from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C, in a 5% CO_2 cell incubator. To validate the effects of HMGB1 on CRISPLD2 expression, THP-1 cells were plated at a final concentration of 10^6 cells/ml and exposed to HMGB1 stimulation. Cells were harvested at 1 h, 3 h, 6 h, 12 h, 24 h and after HMGB1 stimulation to determine the expression of CRISPLD2. To explore whether the transcription of inflammatory factors were influenced by CRISPLD2, THP-1 cells were treated with HMGB1 (0.1 ug/ml) in combination with recombinant CRISPLD2 (5 ug/ml), or stimulated with HMGB1 (0.1 ug/ml) after knocking down the endogenous CRISPLD2 using siRNA.

RNA interference: The siRNAs were designed and synthesized by a commercial company (Shanghai ShengGong Co., Ltd., Shanghai, China). The target sequences against CRISPLD2 were as follows: Sense: 5'-GAA AGC UCG UCU AGC AUA UTT-3' and antisense: 5'-AUA UGC UAG ACG AGC UUU CTT-3'. For RNA interference, THP-1 cells were seeded in 12-well plates at a density of 10^6 cells/ml and transfected with siRNA using Lipofectamine® 3000 according to the manufacturer's instructions. Cells were harvest for designated experiments after incubation with siRNA for 48 hours.

Real-time quantitative polymerase chain reaction (RTqPCR): Real-time PCR was used to measure mRNA transcription of cytokines (TNF-α, IL-6, IL-8, and MCP-1) and CRISPLD2 in THP-1 cells. All the primers were designed by Oligo 6.71 or identified from the literature previously published studies (**Table 1**). A total of 20 μL reaction system was prepared which contains 0.2 μL SYBER Green (Roche Ltd., Basel, Switzerland), 10 μL PCR Master Mix (TaKaRa, Otsu, Japan), 5 μM of sense and anti-sense primer (1 μL), and 2 μL cDNA, and MQ water for supplement. The procedure for real-time PCR were set as follows: initial pre-denaturation at 96°C for 6 minutes followed by denaturation at 96°C for 20 seconds, annealing at optimal temperature for 30 seconds and continued for 40 cycles. Relative changes in gene expression were calculated using the ΔΔCT method with normalization to β-actin.

Western blot analysis: RIPA lysis buffer containing protease inhibitor cocktail was used to extract total protein from THP-1 cells. The protein concentration of each sample was determined by the BCA Protein Assay Kit. Approximately 50 μg of protein was separated in 10% SDS/PAGE gels under reducing conditions and transferred to polyvinylidene fluoride membranes. Subsequently, the non-specific sites were blocked with 5% skim milk. The membranes were incubated with primary antibodies

and diluted at a ratio of 1:800 in primary antibody dilution buffer at 4°C overnight. Next, the membranes were incubated with HRP-conjugated secondary antibodies and diluted at a ratio of 1:2000 in 2% PBS for 1 hour at room temperature. β-actin was used as a loading control and the protein signals were finally detected using the enhanced chemiluminescence western blotting detection system (Santa Cruz) and analyzed using image J software, the original Western blot bands were shown in Figures S1 and S2.

Statistical analysis

Statistical analyses and data visualization were performed using SPSS 22.0, GraphPad Prism 8, and R software (version 3.6.2). One-way ANOVA (two tailed) or Wilcoxon and Mann-Whitney were used for comparing the differences between two or more groups, as appropriate. Kaplan-Meyer plot and log-rank test were used for survival analysis. The Pearson correlation coefficient was used as a metric for gene co-expression analyses. *P* value < 0.05 was considered to be statistical significance.

Results

HMGB1 is among the differentially expressed genes (DEGs) between septic survivors and non-survivors

After data pre-processing, gene expression profiles of whole blood samples from sepsis patients were obtained from GSE26440 dataset (81 survivors and 17 non-survivors) and GSE95233 dataset (34 survivors and 17 non-survivors). The initial gene expression values were normalized and achieved consistency between arrays (GSE26440 **Figure 1A**, **1C**; GSE95233 **Figure 1B**, **1D**). We identified 123 differentially expressed genes (DEGs, 109 up-regulated, 16 down-regulated) between septic survivors and non-survivors in GSE26440 dataset (**Figure 1E**) and 204 DEGs (72 up-regulated, 132 down-regulated) in GSE95233 dataset (**Figure 1F**). HMGB1 was among the up-regulated genes in both of the two datasets. Moreover, the higher levels of HMGB1 in non-survivors than in survivors were also confirmed by violin plot (**Figure 1G**, **1H**).

CRISPLD2 is positively correlated with HMGB1 in most cancer types and normal tissues

Gene expression profiles involving 7801 tumor samples across 33 cancer types and 7858 normal tissue samples across 31 tissue types were retrieved from TCGA database and GTE database, respectively. CRISPLD2 showed a positive correlation in mRNA expression with HMGB1 in most cancer types (**Figure 2A**), and this correlation was more evident in different normal tissues (**Figure 2B**), suggesting a possible dependency between HMGB1 and CRISPLD2 expression.

CRISPLD2 expression is induced by HMGB1 stimulation in THP-1 cells

THP-1 cells were first treated by HMGB1 with a concentration of 10 ug/ml, as previously described (Signaling of High Mobility Group Box 1 (HMGB1) through Toll-like Receptor 4 in Macrophages Requires CD14). As shown in **Figure 3A**, the expression of CRISPLD2 gradually increased over time and reached a peak at 24 h, which was about 1.6 times higher than the baseline level ($P < 0.01$). Subsequently, we treated THP-1 cells with lower concentrations of HMGB1 (1 ug/ml and 0.1 ug/ml, respectively), and still found that the expression of CRISPLD2 was increased in a time-dependent manner and reached the peak at 24 h, which was about 1.3 and 1 times higher than the baseline level, respectively (**Figure 3B**, **3C**, $P < 0.01$ for all). Next, we explored whether the transcription of CRISPLD2 was also correspondingly increased under the stimulation of HMGB1. We treated THP-1 cells with HMGB1 at a low concentration (0.1 ug/ml) and found that the transcription of CRISPLD2 was also increased in a time-dependent manner, reaching the maximum at 24 h, which was about 1.7 times than the baseline level ($P < 0.01$) (**Figure 3D**). The increased expression of CRISPLD2 triggered by HMGB1 stimulation can be partially reversed by LPS-RS, an antagonist of Toll-like receptor 4 (TLR4), indicating that CRISPLD2 expression is TLR4 dependent (**Figure 4**).

CRISPLD2 suppresses the inflammation triggered by HMGB1 in-vitro

The knockdown of CRISPLD2 or stimulating THP-1 cells with HMGB1 (0.1 ug/ml) was associated with an increased transcription of inflammatory cytokines, including TNF-α, IL-6, IL-8 and MCP-1 (**Figure 5**). In addition, when TPH-1 cells were conditioned by HMGB1 (0.1 ug/ml) stimulation and CRISPLD2 silencing simultaneously, the mRNA synthesis of TNF-α,

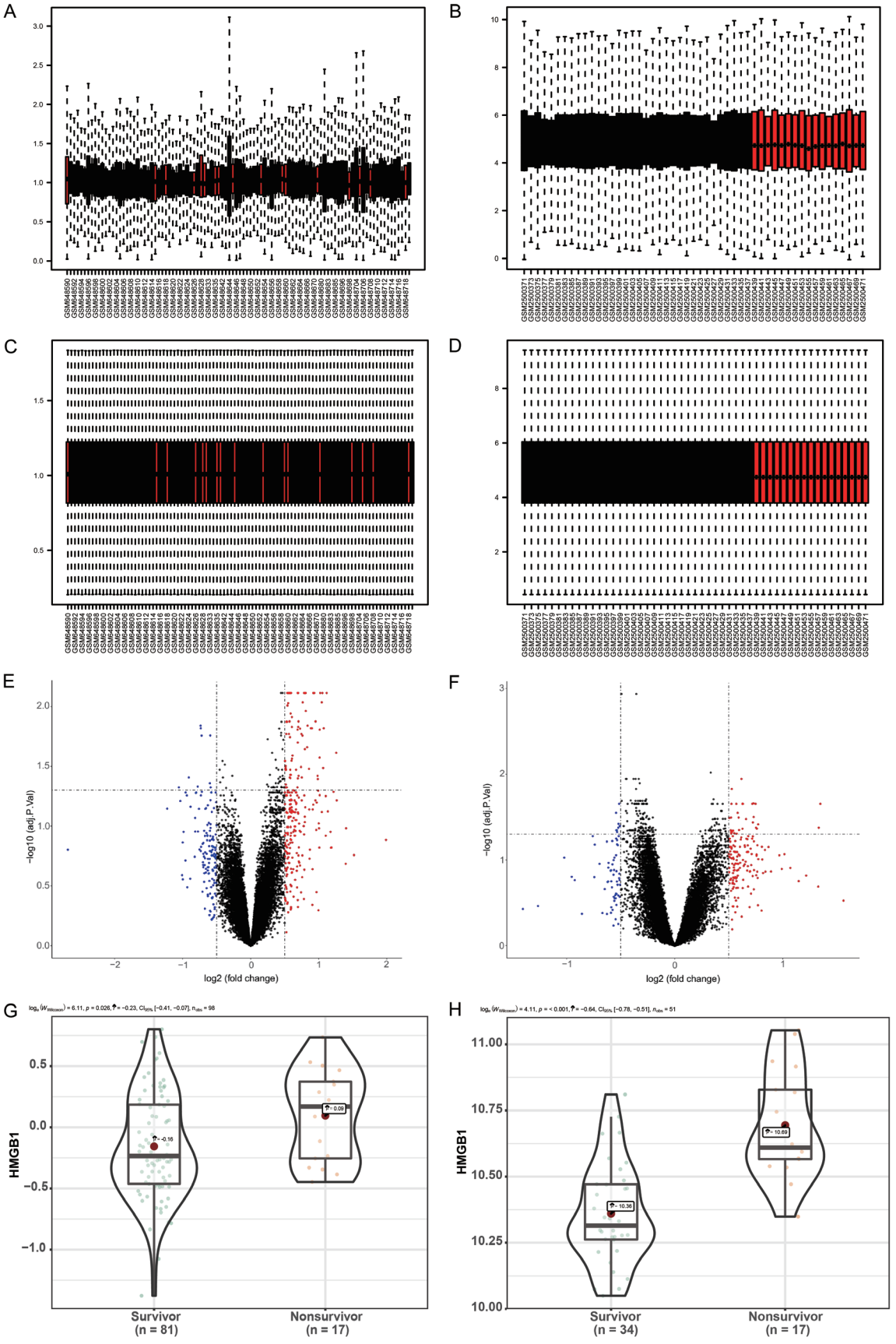
A
B
C
D
E
F
G
H
−log10 (adj.P.Val)
log2 (fold change)
HMGB1
Survivor
(n = 81)
Nonsurvivor
(n = 17)
Survivor
(n = 34)
Nonsurvivor
(n = 17)

Figure 1. (A-D) Normalization of gene expression data. (A, C) and (B, D) represents the gene expression data of GSE26440 and GSE95233 before and after normalization, respectively. (E, F) Volcano plots for differentially expressed genes of GSE26440 and GSE95233 datasets. The red points represent upregulated genes identified using the criteria that |logFC| > 0.1 and Bonferroni-adjusted P < 0.05. The blue points represent downregulated genes identified using the same criteria. The black points represent genes with non-significant changes in gene expression. (G, H) Violin plots of HMGB1 expression in septic survivors and non-survivors of GSE26440 and GSE95233 datasets.

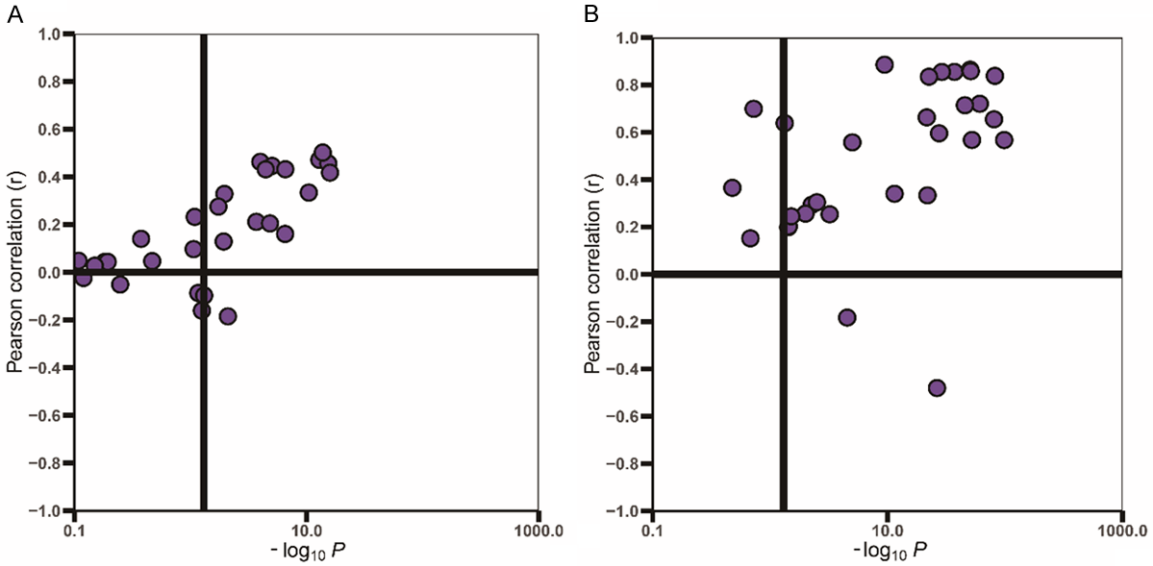

Figure 2. The correlation of HMGB1 and CRISPLD2 across 33 cancer types and 31 normal tissue types. Each point represents one cancer tissue type (A) or one normal tissue type (B).

IL-6, IL-8 and MCP-1 was more evident (**Figure 5**). We next explored whether HMGB1 triggered inflammatory cytokines transcription was reversed by exogenous recombinant CRISPLD2. As shown in **Figure 6**, HMGB1 treated THP-1 cells presented enhanced transcription of inflammatory cytokines (TNF-α, IL-6, IL-8 and MCP-1), and the pro-inflammatory effects of HMGB1 was partially reversed by CRISPLD2 administration. Intriguingly, the baseline level of inflammatory cytokines transcription was also slightly down-regulated by CRISPLD2 administration, although the effect was not significant.

CRISPLD2 reduces serum levels of inflammation in septic mice

To verify whether the anti-inflammatory effect of CRISPLD2 can be reproduced in vivo, we induced sepsis in mice using the cecal ligation and puncture (CLP) method and intravenously injected mice with recombinant CRISPLD2 at a therapeutic dose (50 mg/kg) 6 hours after CLP surgery. Compared with mice that underwent sham surgery, mice that received CLP surgery showed a significant higher level of serum TNF-α and IL-6. However, the elevated serum TNF-α and IL-6 which was induced by CLP surgery was partially reduced by intravenous treatment of recombinant CRISPLD2 (**Figure 7**).

CRISPLD2 treatment fails to provide survival benefit in mice with sepsis

Given that one of the important pathophysiological characteristics of sepsis is excessive inflammatory response, we further explored whether the anti-inflammatory profile of CRISPLD2 in vivo could provide a survivor benefit for septic mice. As shown in **Figure 8**, septic mice that received recombinant CRISPLD2 (50 mg/kg) treatment intravenously presented similar survival curve as compared to those that received equivalent volume of normal saline. The 10-day survival rates were 30% for the CRISPLD2 treatment group and 25% for the control group, respectively (P=0.732).

Discussion

The pathogenesis of sepsis is extremely complex, which often involves an excessive inflam-

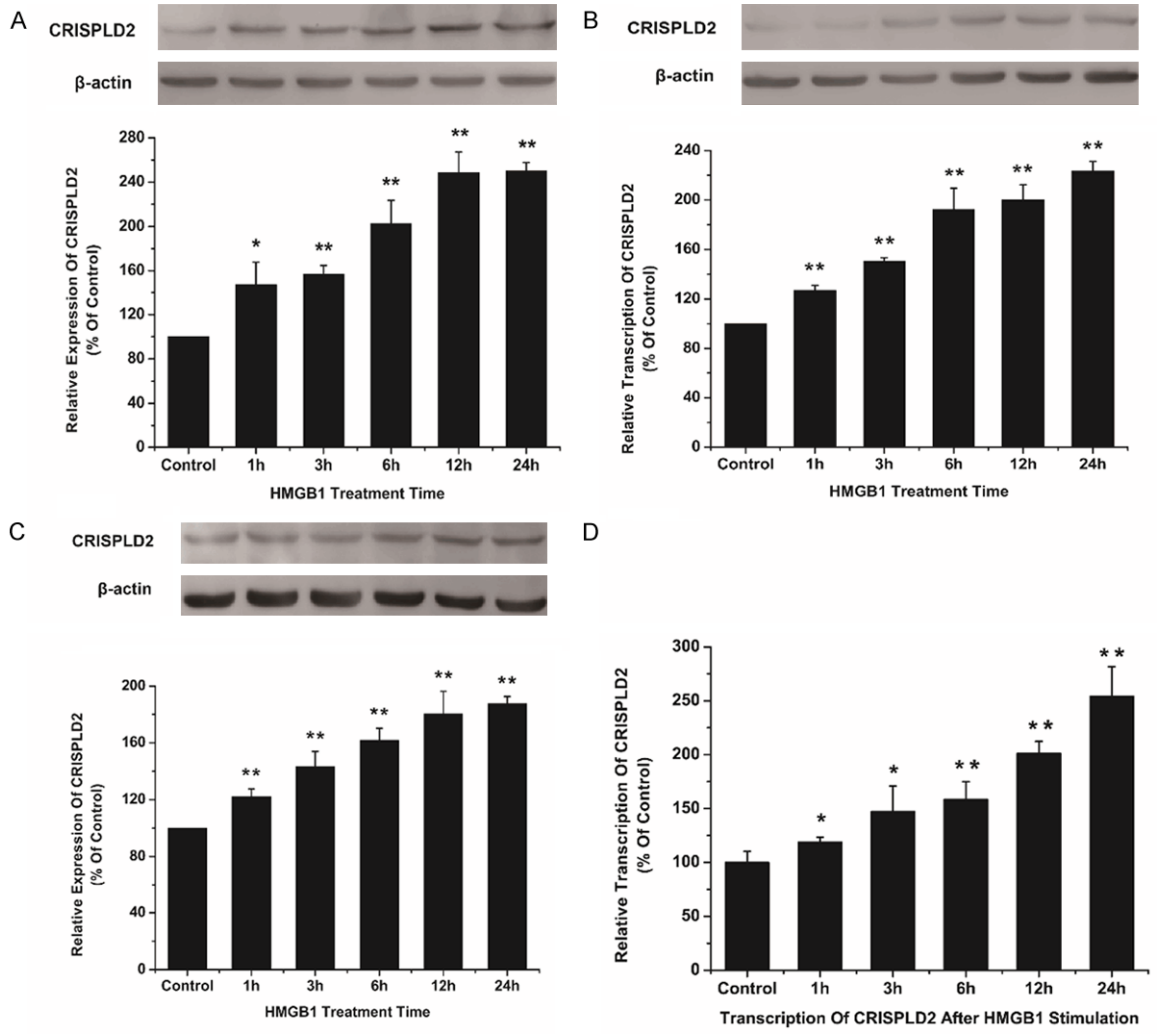

Figure 3. Expression and transcription of CRISPLD2 in THP-1 cells under the stimulation of HMGB1 at different concentrations. (A-C) Expression of CRISPLD2 when THP-1 cells were treated by HMGB1 at a final concentration of 10 ug/ml (A), 1 ug/ml (B), and 0.1 ug/ml (C). (D) mRNA synthesis of CRISPLD2 when THP-1 cells were treated by HMGB1 at a final concentration of 0.1 ug/ml. *P < 0.05 compared with the control group. **P < 0.01 compared with the control group.

matory response to infection, followed by tissue injuries, organ dysfunctions, and even death. To ingest and clear invading pathogens and microbial products during sepsis, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) were detected by the monocytes/macrophages and other phagocytes, such as neutrophils [9]. PAMPs, such as bacterial peptidoglycan, DS-RNA, endotoxin, bind to macrophage/monocyte receptors (TLR-2, 3, 4, and 9) [10-14], triggering sequential release of pro-inflammatory mediators in the early stage (TNF, IL-1, and IFN-γ) and in the late stage (HMGB1) [3, 15]. However, it is difficult to intervene in the early inflammatory cytokine accumulation in clinical settings because the time interval between pathogens invasion and inflammatory cascade initiation is usually very short [16]. This prompts us to search for other potential targets, such as HMGB1, which may provide us with a broader therapeutic window.

CRISPLD2 is a cystine-rich secretory protein (CRISP) with a secretory signal and two LCCL domains. The unique structure of two LCCL domains has significant lipopolysaccharides (LPS)-binding affinity. Therefore, CRISPLD2 have anti-endotoxin effects and protect mice from endotoxin shock [7]. Correspondingly, low

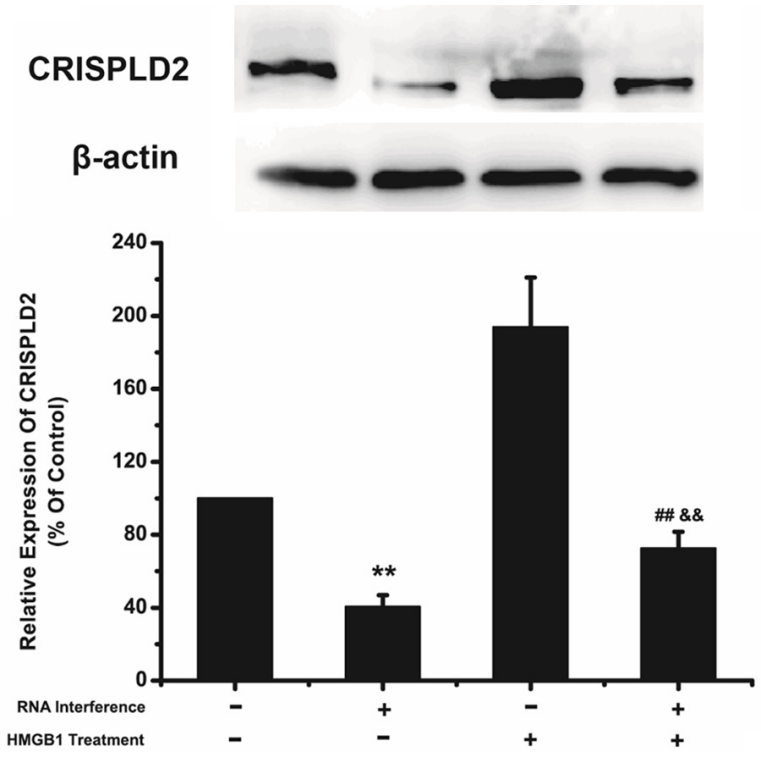

Figure 4. Effects of TLR4 blockade on CRISPLD2 expression. LPS-RS (TLR4 antagonist) and HMGB1 (0.1 ug/ml) were added into THP-1 cells as indicated. CRISPLD2 expression was detected after 24 h. **P < 0.01 compared with the control group. ##P < 0.01 compared with the LPS-RS group. &&P < 0.01 compared with the HMGB1 group.

serum CRISPLD2 level may predict a poor outcome in patients with sepsis or septic shock [17]. In addition, CRISPLD2 also exhibited a broad range of anti-inflammatory properties without the presence of LPS in a series of inflammation related disease, including hepatic fibrogenesis [18], asthma [19], gaucher disease [20], and obesity [21], suggesting that the anti-inflammatory activity of CRISPLD2 is not only restricted to its interaction with LPS. In a previous study, Zhang et al. found that CRISPLD2 can alleviate HMGB1 induced inflammation in hepatic fibroblast cells. Therefore, we hypothesized that CRISPLD2 may also serve as an anti-inflammatory component targeting HMGB1 [18].

In our study, we first conducted bioinformatic analysis to explore the relationship between HMGB1 and CRISPLD2. We found that septic non-survivors had a significant higher level of serum HMGB1 than septic survivors. This finding is consistent with previous studies, which suggested that HMGB1 acted a late mediator of systematic inflammation and lethality in sepsis [22]. We also found that CRISPLD2 positively correlated with HMGB1 in mRNA expression in most of the cancer and normal tissue types, revealing a co-expression or dependency relationship between the two genes, even without any external stimuli. Subsequently, using cultured THP-1 cells, we confirmed that HMGB1 can induce the expression of CRISPLD2 in a time dependent manner through TLR4-dependent pathway. Given that CRISPLD2 and HMGB1 shared a wide range of time scales in gene expression and the anti-inflammatory property of CRISPLD2, we further assumed that HMGB1 induced cytokines production might be partially reversed by CRISPLD2 and verified this assumption both in vitro and in vivo.

Although our findings suggested that CRISPLD2 could inhibit the inflammatory cytokines produced by HMGB1, the exact mechanism that how CRISPLD2 mediates this effect remains unclear. In a previous study, Wang et al. reported a pattern by which CRISPLD2 alleviated LPS induced inflammation. In brief, soluble CRISPLD2 could serve as a LPS-binding protein to competitively inhibit the interaction of LPS with its target receptor (TLR4), thereby partially blocked the inflammatory cascade triggered by LPS. Although we are still unclear the CRISPLD2 mediated anti-inflammatory pattern is only unique to LPS or equal to any other stimulatory agents (e.g. HMGB1), it is more reasonable to suppose that CRISPLD2 interacts with ligands rather than receptors since the previous study failed to demonstrate a direct binding between CRISPLD2 and immune cell surface, where diverse receptors intensively expressed. In addition, unlike other traditional ligands, HMGB1 can interact with a variety of company molecular (including LPS, Nucleic acids, Pam3CSK4, IL-1a/b, and Nucleosome) and form HMGB1-partner complexes that can regulate the magnitude of immune responses [16]. One of our future stud-

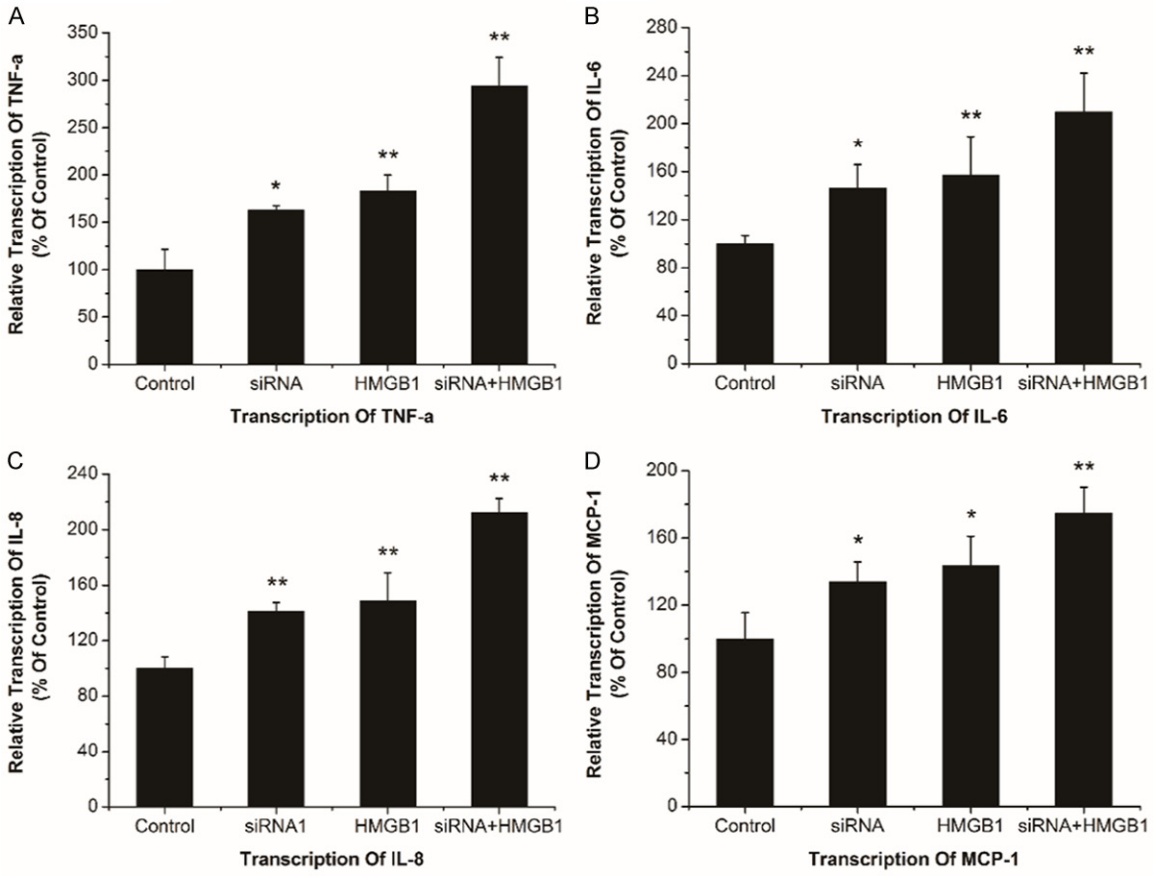

Figure 5. Effects of CRISPLD2 interference on inflammatory cytokines transcription in THP-1 cells. The four groups were control group, CRISPLD2 interference group, HMGB1 stimulation group (0.1 ug/ml), and HMGB1 stimulation with CRISPLD2 interference group. A. Transcription of TNF-α. B. Transcription of IL-6. C. Transcription of IL-8. D. Transcription of MCP-1. *P < 0.05 compared with the control group. **P < 0.01 compared with the control group.

ies is to explore whether there is any interaction or linkage between CRISPLD2 and HMGB1, and whether the interaction, if exist, is involved in mediating the down-regulation of HMGB1 triggered inflammatory responses. Another interesting finding of our study was that CRISPDL2 not only abbreviated LPS or HMGB1 induced pro-inflammatory cytokines production, but also shifted the baseline cytokines production to a lower level in the absence of any stimuli, although this effect was not significant. This indicates even without any immunostimulation, CRISPLD2 can still actively participated in modeling the immune state through totally unknown pathway.

Finally, we explored the therapeutic potential of CRISPLD2 as an anti-inflammatory agent targeting HMGB1 in septic mice. Unfortunately, intravenously treatment of CRISPLD2 failed to rescue septic mice. The failure of using CRISPLD2 as an anti-inflammatory agent for treating sepsis emphasizes the complexity of sepsis, which is far beyond inflammation. For instance, even HMGB1 itself has multiple roles in the pathogenesis of sepsis, including the involvement in cellular autophagy [23], pyroptosis [24], and neutrophil extracellular traps (NETs) [25]. Therefore, only targeting the inflammation induced by HMGB1 is insufficient to provide survival benefit.

In conclusion, our study demonstrated that HMGB1 can act as stimuli to up-regulate the expression of CRISPLD2 in THP-1 cells, and in turn, increased CRISPLD2 can curtail HMGB1 induced pro-inflammatory cytokines production. However, the anti-inflammatory effects of CRISPLD2 did not translate into survival benefit in mice with sepsis.

Acknowledgements

We would like to thank all the participants involved in this study. This work was supported by the National science Foundation of china

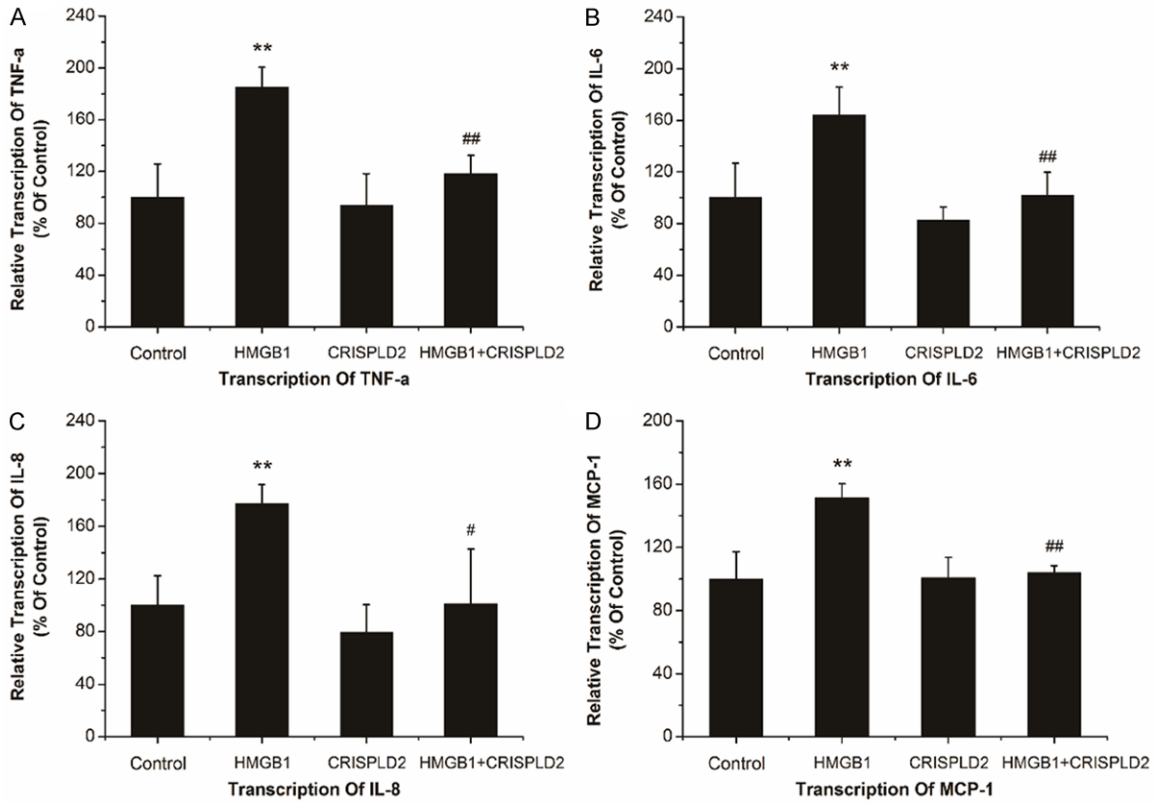

Figure 6. Effects of exogenous recombinant CRISPLD2 administration on inflammatory cytokines transcription in THP-1 cells. The four groups were control group, HMGB1 stimulation group (0.1 ug/ml), CRISPLD2 administration group (5 ug/ml), HMGB1 stimulation group (0.1 ug/ml) and CRISPLD2 administration group (5 ug/ml). A. Transcription of TNF-α. B. Transcription of IL-6. C. Transcription of IL-8. D. Transcription of MCP-1. *P < 0.05 compared with the control group. **P < 0.01 compared with the control group. ##P < 0.05 compared with HMGB1 group. ##P < 0.01 compared with HMGB1 group.

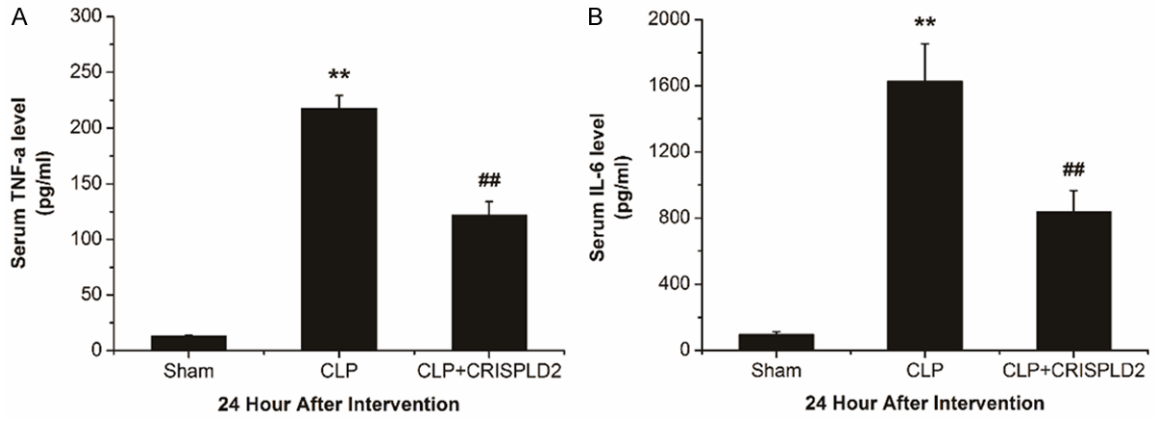

Figure 7. Effects of recombinant CRISPLD2 on serum inflammatory cytokine in septic mice. Mice received sham surgery, CLP surgery, and CLP surgery followed by intravenous treatment of recombinant CRISPLD2 (50 mg/kg, 6 hours after CLP surgery), respectively. The levels of TNF-α (A) and IL-6 (B) in serum were detected 24 h after operation. Each group contained measurements from three mice. **P < 0.01 compared with the control group. ##P < 0.01 compared with the control group.

(No. 81571942) and Science and Technology Commission of Shanghai Municipality (No. 19495810200, No. 18DZ2251200 and No. 18411951400).

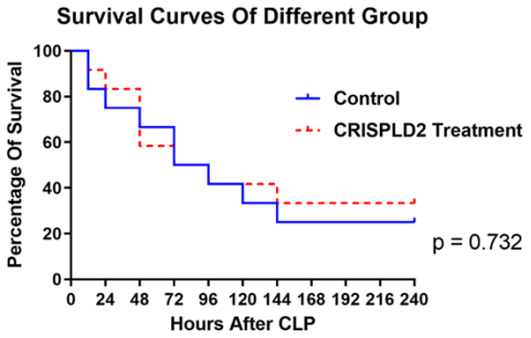

Figure 8. Effects of recombinant CRISPLD2 administration on survival in mice with sepsis. The red dotted line represents the survival curve of CRISPLD2 treatment group (n=12, 50 mg/kg), and the solid blue line represents the survival curve of control group (n=12, the same volume of normal saline).

Disclosure of conflict of interest

None.

Address correspondence to: Dechang Chen, Department of Critical Care Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. Tel: +86-18918520002; E-mail: chendechangsh@hotmail.com; Drs. Zhaofen Lin and Wenfang Li, Department of Critical Care Medicine, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China. Tel: +86-13601605100; E-mail: linzhaofen@hotmail.com (ZFL); Tel: +86-13501838919; E-mail: chzhedlwf@163.com (WFL)

References

[1] Russell J. Management of sepsis. N Engl J Med 2006; 355: 1699-1713.

[2] Bhatia M, He M, Zhang H and Moochhala S. Sepsis as a model of SIRS. Front Biosci (Landmark Ed) 2009; 14: 4703-4711.

[3] Wang H, Bloom O, Zhang M, Vishnubhakat J, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue K, Faist E, Abraham E, Andersson J, Andersson U, Molina P, Abumrad N, Sama A and Tracey K. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285: 248-251.

[4] Andersson U and Tracey K. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011; 29: 139-162.

[5] Wang X, Xiang L, Li H, Chen P, Feng Y, Zhang J, Yang N, Li F, Wang Y, Zhang Q, Li F and Cao F. The role of HMGB1 signaling pathway in the development and progression of hepatocellular carcinoma: a review. Int J Mol Sci 2015; 16: 22527-22540.

[6] Ivanov S, Dragoi A, Wang X, Dallacosta C, Louten J, Musco G, Sitia G, Yap G, Wan Y, Biron C, Bianchi M, Wang H and Chu W. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood 2007; 110: 1970-1981.

[7] Wang Z, Xing W, Fan H, Wang K, Zhang H, Wang Q, Qi J, Yang H, Yang J, Ren Y, Cui S, Zhang X, Liu F, Lin D, Wang W, Hoffmann M and Han Z. The novel lipopolysaccharide-binding protein CRISPLD2 is a critical serum protein to regulate endotoxin function. J Immunol 2009; 183: 6646-6656.

[8] Rittirsch D, Huber-Lang M, Flierl M and Ward P. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc 2009; 4: 31-36.

[9] Luster A, Alon R and von Andrian U. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol 2005; 6: 1182-1190.

[10] Ha T, Lu C, Liu L, Hua F, Hu Y, Kelley J, Singh K, Kao R, Kalbfleisch J, Williams D, Gao X and Li C. TLR2 ligands attenuate cardiac dysfunction in polymicrobial sepsis via a phosphoinositide 3-kinase-dependent mechanism. American journal of physiology. Am J Physiol Heart Circ Physiol 2010; 298: H984-91.

[11] Gao M, Ha T, Zhang X, Liu L, Wang X, Kelley J, Singh K, Kao R, Gao X, Williams D and Li C. Toll-like receptor 3 plays a central role in cardiac dysfunction during polymicrobial sepsis. Crit Care Med 2012; 40: 2390-2399.

[12] Brightbill H, Libraty D, Krutzik S, Yang R, Belisle J, Bleharski J, Maitland M, Norgard M, Plevy S, Smale S, Brennan P, Bloom B, Godowski P and Modlin R. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999; 285: 732-736.

[13] Poltorak A, He X, Smirnova I, Liu M, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B and Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282: 2085-2088.

[14] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K and Akira S. A toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-745.

[15] Chan E and Riches D. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 (mapk) in a mouse macrophage cell line. Am J Physiol Cell Physiol 2001; 280: C441-C450.

[16] Wang H, Ward M and Sama A. Targeting HMGB1 in the treatment of sepsis. Expert Opin Ther Targets 2014; 18: 257-268.

[17] Wang T, Wang Z, Wang L, Yan L, Wan J, Zhang S, Jiang H, Li W and Lin Z. CRISPLD2 is ex-

pressed at low levels during septic shock and is associated with procalcitonin. PLoS One 2013; 8: e65743.
[18] Zhang H, Liu Z and Liu S. HMGB1 induced inflammatory effect is blocked by CRISPLD2 via MiR155 in hepatic fibrogenesis. Mol Immunol 2016; 69: 1-6.
[19] Yan D, Hamed O, Joshi T, Mostafa M, Jamieson K, Joshi R, Newton R and Giembycz M. Analysis of the indacaterol-regulated transcriptome in human airway epithelial cells implicates gene expression changes in the adverse and therapeutic effects of β2-adrenoceptor agonists. J Pharmacol Exp Ther 2018; 366: 220-236.
[20] Lugowska A, Hetmańczyk-Sawicka K, Iwanicka-Nowicka R, Fogtman A, Cieśla J, Purzycka-Olewiecka J, Sitarska D, Płoski R, Filocamo M, Lualdi S, Bednarska-Makaruk M and Koblowska M. Gene expression profile in patients with Gaucher disease indicates activation of inflammatory processes. Sci Rep 2019; 9: 6060.
[21] Jackson R, Griesel B, Short K, Sparling D, Freeman W and Olson A. Weight loss results in increased expression of anti-inflammatory protein CRISPLD2 in mouse adipose tissue. Obesity (Silver Spring) 2019; 27: 2025-2036.
[22] Wang H, Yang H, Czura C, Sama A and Tracey K. HMGB1 as a late mediator of lethal systemic inflammation. Am J Respir Crit Care Med 2001; 164: 1768-1773.
[23] Qu L, Chen C, Chen Y, Li Y, Tang F, Huang H, He W, Zhang R and Shen L. High-mobility group box 1 (HMGB1) and autophagy in acute lung injury (ALI): a review. Med Sci Monit 2019; 25: 1828-1837.
[24] Paterson C, Ford M and Coopersmith C. Breaking the bond between tetranectin and HMGB1 in sepsis. Sci Transl Med 2020; 12: eabb2575.
[25] Cheng Z, Abrams S, Toh J, Wang S, Wang Z, Yu Q, Yu W, Toh C and Wang G. The critical roles and mechanisms of immune cell death in sepsis. Front Immunol 2020; 11: 1918.

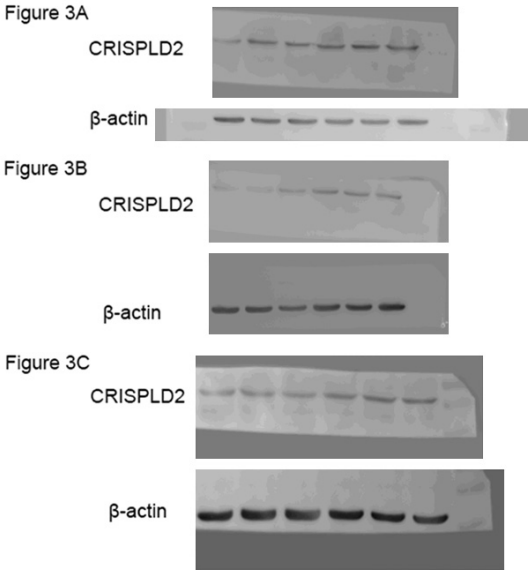

Figure S1. The original **Figure 3** of Western Blot.

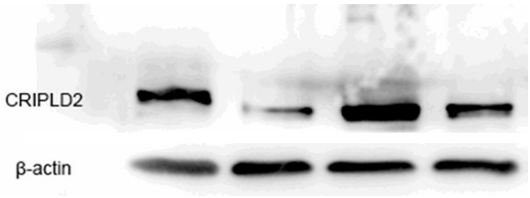

Figure S2. The original **Figure 4** of Western Blot.