Linkage of lncRNA CRNDE sponging miR-181a-5p with aggravated inflammation underlying sepsis

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Abstract

This investigation was performed to verify whether lncRNA CRNDE sponging miR-181a-5p was involved with sepsis-relevant inflammatory dysfunctions. Aggregately 136 sepsis patients and 151 healthy people were recruited, and their fasting peripheral blood was gathered to detect expressions of CRNDE and miR-181a-5p. In addition, THP-1 cells were transfected with si-CRNDE, miR-181a-5p mimic, pcDNA3.1-TLR4 and si-TLR4, and then sepsis-specific inflammatory cytokines within the cells were quantified. The sponging relationships between CRNDE and miR-181a-5p, as well as between miR-181a-5p and TLR4, were ascertained by means of luciferase reporter gene assay. The experimental results revealed that over-expressed CRNDE and under-expressed miR-181a-5p were associated with shortened lifespan of sepsis patients. Mechanically, si-CRNDE-1 and miR-181a-5p mimic were able to reverse the promoting effects of LPS on production of NF-kB, TNF-α, IL-1β and IL-6 by THP-1 cells. Moreover, the expressional change of miR-181a-5p in THP-1 cells was in part owing to its being sponged by CRNDE. Lastly, TLR4, subjected to targeted modification of miR-181a-5p, was capable of disturbing the contribution of CRNDE and miR-181a-5p to THP-1 cells' release of NF-kB, TNF-α, IL-1β and IL-6. Collectively, the CRNDE/miR-181a-5p/TLR4 axis seemed to have potential in modifying sepsis-related inflammatory pathogenesis, which offered a direction for sepsis diagnosis and treatment.

Introduction

Sepsis, a systemic inflammatory response syndrome, usually occurs along with severe trauma, burns, shock and severe surgery, and it is clinically featured by massive release of inflammatory mediators into peripheral blood.[1,2] The mortality caused by severe sepsis is expected to achieve 28–50%,[3,4] and more unfortunately, patients with a dysfunctional cardiovascular system are more prone to death when they were complicated by sepsis.[5–7] Hence, it is of utmost importance to clarify root causes that explain sepsis onset, and also biomarkers for the progression of sepsis. Acknowledging the fundamental role of pro-inflammatory cytokines in boosting sepsis development,[8] exploring biomarkers that powerfully modify inflammatory responses might be beneficial to uncovering sepsis etiology.

To date, sepsis-focused research has been mostly related to protein-coding genes, rather than non-coding RNAs, such as long non-coding RNAs (lncRNAs), that fail to encode proteins.[9,10]

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Nonetheless, the involvement of lncRNAs in immune reactions has been increasingly revealed,[11–13] and the immune-modulatory effects of lncRNAs have also been confirmed in various immune cells, such as monocytes and dendritic cells.[14] Furthermore, profiling of lncRNAs showed discrepant patterns during differentiation of Th1, Th2 and Th17 cells,[15] which further highlighted the high-level involvement of lncRNAs in inflammation. Notable, lncRNA colorectal neoplasia differentially expressed (CRNDE), according to results derived from Kyoto Encyclopedia of Genes and Genomes (KEGG) database, was conjectured as an immunity-relevant gene, and a majority of its functions were centered on regulation of immune responses, such as TLR signaling, TNF-α signaling and the cytokine–cytokine receptor interaction pathway.[16] The *in vitro* experiments also supported the role CRNDE played in activation of MyD88-independent TLR signaling and subsequent NF-κB signaling,[16] whose normal functioning appeared fundamental for restraint of sepsis development.[17–20] As a consequence, it is plausible that CRNDE could play a part in the acceleration of sepsis aggravation. In addition, miR-181a-5p was negatively modified by CRNDE, and the Wnt/β-catenin signaling regulated by it served as a powerful mediator underlying sepsis-correlated inflammation.[21,22] In addition, the activity of NF-κB could be undermined after stimulation of over-expressed miR-181a,[23] even though whether the inflammation-boosting capacity of NF-κB would be weakened by miR-181a remained uncertain. Furthermore, miR-181a-5p was additionally supposed as a modulator of immune responses in dendritic cells via negative modification of TNF-α.[24] In summary, CRNDE and miR-181a-5p were tightly linked with inflammatory disorders inherent in sepsis etiology; nonetheless, whether CRNDE combined with miR-181a-5p indeed promoted or delayed sepsis development was still unknown.

In order to fill this knowledge gap, this investigation was designed to explore the inner linkage of CRNDE with miR-181a-5p in sepsis, which might guide fruitful diagnosis and treatment for sepsis in future.

Materials and methods

Inclusion of clinical samples

In total 136 sepsis patients aged between 18 and 76 yr, were recruited from the respiratory department of Chenzhou No.1 People's Hospital, and their confirmed diagnoses were in accordance with the criteria formulated by American College of Chest Physicians and Critical Care Medicine in 2012.[25] Sepsis cases were excluded when they were found with symptoms related with immune system disorders, hematological diseases, chronic organ dysfunctions, seriously impaired hepato-renal functions and cancers. In the meantime, 151 healthy people who took physical examinations in the hospital were incorporated into the control group. The fasting peripheral blood samples collected from patients with sepsis and healthy controls in the morning were centrifuged at 594 g for 10 min, after which the supernatants were stored at –80°C. All procedures required by this investigation have been approved by Chenzhou No.1 People's Hospital and the ethics committee of Chenzhou No.1 People's Hospital. All the participants and their relatives have signed informed consents after being fully notified.

Cell treatment and cell transfection

The THP-1 cell line, a human myeloid leukemia mononuclear cell line, was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were incubated in the atmosphere of 5% CO_2 and 37°C, and their culture medium was managed as DMEM complete medium that contained 10% FBS.[26]

The THP-1 cells at the logarithmic phase were seeded at the concentration of 5.0×10^6/ml, and cell models of sepsis were built by stimulating the THP-1 cell line with 100 ng/ml LPS for 4 h.[27] Also, the THP-1 cells that grew to 70%-80% confluence were transfected with siRNAs against CRNDE, si-NC, miR-181a-5p mimic, miR-NC and si-TLR4 (Genepharma, China) (Supplemental Table 1). After 48 h, the cells were used for the following experiments.

RT-PCR

The total RNAs were extracted from blood or cells according to instructions of the Trizol kit (Invitrogen, USA), and they were then dissolved within diethylpyrocarbonate (DEPC)-treated distilled water. The concentration and quality of RNAs were measured using a spectrophotometer (model: Nano Drop 2000, Applied Biosystems, USA), and their quality was satisfied when the A260/A280 ratio ranged between 1.9 and 2.1. Afterward, the collected RNAs were reversely transcribed into cDNAs, aided by the PrimeScript™ RT reagent kit (Takara, Japan). Then with primers synthesized by Sangon (Shanghai, China) (Table 1), a PCR reaction was implemented on a PCR instrument (model: Applied Biosystems 7600, ABI, USA), as per the instructions of SYBR Premix ExTaq II Kit (Takara, Japan). The reaction steps were pre-denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 5 s and extension at 72°C for 30 s. Finally, expression of target genes was

Table 1. Primers used in RT-PCR analysis.

Gene	Direction	Primer sequences (5'-3')
CRNDE	Forward	CGCGCCCGCGCGGCGGAGGA
	Reverse	TATGAATTGCAGACTTTGCAGA
miR-181a-5p	Forward	GCCGAACATTCAACGCTGTCG
	Reverse	GTGCAGGGTCCGAGGT
TLR4	Forward	TGAGCAGTCGTGCTGGTATC
	Reverse	CAGGGCTTTTCTGAGTCGTC
NF-κB	Forward	CAGAGGGACAACAGCAATGA
	Reverse	CCGTGTAAACCAAAGCCTA
TNF-α	Forward	CCCTCCCCATGGAGCCAGCT
	Reverse	GCACAGAGGCCAGGGGGCTA
IL-1β	Forward	TCAGGCAGGCAGTATCACTC
	Reverse	GCAAGGTCCACGGGAAAGAC
IL-6	Forward	TCGAGCCCACCGGGAACGAA
	Reverse	GCAACTGGACCGAAGGCGCT
GAPDH	Forward	GTCAACGGATTTGGTCTGTATT
	Reverse	AGTCTTCTGGGTGGCAGTGAT
U6	Forward	GTGCTCGCTTCGGCAGCACAT
	Reverse	ATGGAACGCTTCACGAATTTG

quantified on the basis of $2^{-\Delta\Delta Ct}$ method, with U6 or GAPDH as the internal reference.

Western blotting

The cells or blood were added with pre-cooled lysis buffer, and the supernatants were taken after 4°C and 9590 g centrifugation for 5 min. The concentration of total protein was detected using the Bradford method, and 40 μg of the protein was used for 10% SDS-PAGE. The samples were then transferred onto a polyvinylidene fluoride (PVDF) membrane via the wet method, and were blocked at 37°C within TBST that contained 5% skim milk powder for 1 h. Subsequently, the samples were incubated within primary Abs (rabbit anti-human, Abcam, USA) against NF-kB (1:1000, Cat. No.: ab32536), TNF-α (1:500, Cat. No.: ab6671), IL-1β (1:1000, Cat. No.: ab2105), IL-6 (1:500, Cat. No.: ab6672), TLR4 (1:500, Cat. No.: ab13556) and GAPDH (1:10000, Cat. No.: ab181602) at 4°C overnight. Then goat anti-rabbit secondary Abs marked with HRP (1:10000, Cat. No.: ab97051, Abcam, USA) were added to the samples for additional 1 h incubation at room temperature. The products were washed five times with TBST, each time for 5 min. After development and exposure in the dark, the Gene Tools software was applied to measure the expression level of target proteins, with GAPDH as the reference.

Luciferase reporter gene assay

The CRNDE and TLR4 sequences that contained specific target sites of miR-181a-5p were connected to psi-

Check2 (Promega, USA), and were then amplified through PCR. The reclaimed products were called psi-Check2-CRNDE Wt and psi-Check2-TLR4 Wt. Meanwhile, psi-Check2-CRNDE Mut and psi-Check2-TLR4 Mut were constructed following identical procedures, except that the CRNDE and TLR4 sequences were mutated in their miR-181a-5p targeting sites. In line with the guidance of Lipofectamine TM2000 kit (Invitrogen, USA), miR-181a-5p mimic and miR-NC were, respectively, co-transfected into cells with psi-Check2-CRNDE Wt, psi-Check2-CRNDE Mut, psi-Check2-TLR4 Wt and psi-Check2-TLR4 Mut. Finally, the dual-luciferase reporter assay kit (Promega, USA) was used to determine the luciferase activity of cells.

Statistical analyses

The data were statistically analyzed using SPSS 13.0 software. The count data expressed as percentage (%) were compared through χ^2 test, and the measurement data presented in the form of mean ± standard deviation (SD) were contrasted using student's t-test. Survival curves were plotted utilizing the Kaplan–Meier method, with log-rank test adopted for between-group comparisons. Statistical significance was deemed at P values of < 0.05.

Results

Comparison of baseline clinical characteristics between sepsis patients and healthy controls

The patients with sepsis and the healthy controls were matched with respect to mean age ($P = 0.130$) and sex ratio ($P = 0.204$) (Table 2). However, significantly heightened levels of white blood cells (WBC), C-reactive protein (CRP) and procalcitonin (PCT) were detected among sepsis patients, when compared with healthy controls ($P < 0.001$). The average MAP, SOFA and APACHE II scores of sepsis patients were 0.34 ± 0.08, 7.64 ± 0.67 and 17.15 ± 1.22, respectively.

Association of CRNDE and miR-181a-5p expressions with clinical traits of sepsis patients

The sepsis patients displayed higher CRNDE expression and lower miR-181a-5p expression than healthy controls ($P < 0.05$) (Figure 1a). In addition, the sepsis patients were divided into over-expressed CRNDE (> median expression) and under-expressed (≤ median expression) CRNDE groups (Table 3). Similarly, the same sepsis patients were categorized into over-expressed miR-181a-5p group and under-expressed miR-181a-5p group, with the median expression of

miR-181a-5p as the dividing point. The results revealed that sepsis patients carrying over-expressed CRNDE and under-expressed miR-181a-5p expression were tracked with higher MAP (≥ 0.34), SOFA (≥ 7.64)

Table 2. Comparison of baseline clinical features between sepsis patients and healthy controls.

Clinical features	Sepsis patients	Healthy controls	t/χ^2 Value	*P* Value
Gender			1.62	0.204
Female	66	62		
Male	70	89		
Age (yr)	56.04 ± 2.03	55.61 ± 2.68	1.52	0.130
MAP score	0.34 ± 0.08	–	–	–
SOFA score	7.64 ± 0.67	–	–	–
APACHE II score	17.15 ± 1.22	–	–	–
WBC ($\times 10^9$/l)	14.37 ± 5.61	7.42 ± 1.31	14.79	< 0.001
CRP (mg/l)	125 ± 39	4.69 ± 1.53	37.88	< 0.001
PCT (µg/l)	9.51 ± 4.52	0.22 ± 0.10	25.25	< 0.001

MAP: mean arterial pressure; SOFA: sequential organ failure assessment; APACHE II: acute physiology and chronic health evaluation II; WBC: white blood cell count; CRP: C-reactive protein; PCT: procalcitonin.

and APACHE II (≥ 17.15) scores, along with larger amounts of WBC ($\geq 14.37 \times 10^9$/l), CRP (≥ 125 mg/l) and PCT (≥ 5.63 µg/l), than those categorized into the under-expressed CRNDE group and over-expressed miR-181a-5p group ($P < 0.05$). Interestingly, there emerged a negative correlation between CRNDE expression and miR-181a-5p expression among the recruited sepsis subjects ($r_s = -0.451$, 95% CI: -0.575 to -0.305, $P < 0.001$) (Figure 1b).

Predictive role of CRNDE and miR-181a-5p for prognosis of patients with sepsis

Over-expressed CRNDE was associated with poorer overall survival than under-expressed CRNDE ($P = 0.001$), while on the contrary, under-expressed miR-181a-5p might prolong the lifespan of patients with sepsis in comparison to over-expressed miR-181a-5p ($P = 0.002$) (Figure 1c). In addition, both CRNDE and miR-181a-5p were identified as significant predictors for the prognosis of sepsis patients, according to results of univariate and multivariate analyses ($P < 0.05$) (Table 4). Concurrently, higher
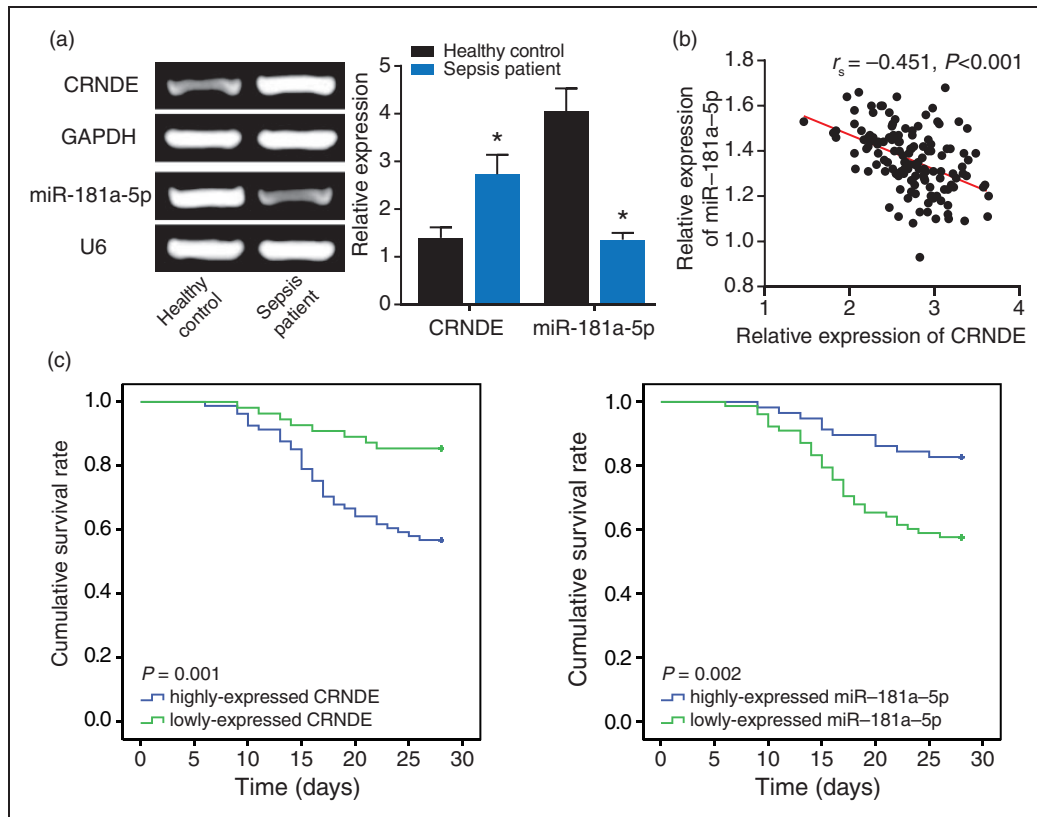

Figure 1. Association of CRNDE and miR-181a-5p expressions with the prognosis of sepsis patients. (a) The CRNDE and miR-181a-5p expressions were compared between sepsis patients and healthy controls. *: $P < 0.05$ when compared with healthy controls. (b) The CRNDE expression was negatively correlated with miR-181a-5p expression among the sepsis patients. (c) The low-expressed CRNDE and high-expressed miR-181a-5p were associated with favorable survival of sepsis patients, with high-expressed CRNDE and low-expressed miR-181a-5p, respectively, as the reference.

Table 3. Association of CRNDE/miR-181a-5p expressions with the baseline clinical features of sepsis patients.

Clinical features	LncRNA CRNDE expression				miR-181a-5p expression			
	High	Low	χ^2 Value	P Value	High	Low	χ^2 Value	P Value
Gender								
Female	35	31			31	35		
Male	46	24	2.27	0.132	27	43	0.98	0.322
Age								
$\geq$ 56.04	39	32			35	36		
< 56.04	42	23	1.32	0.250	23	42	2.69	0.101
MAP score								
$\geq$ 0.34	62	30			31	61		
< 0.34	19	25	7.24	0.007	27	17	9.32	0.002
SOFA score								
$\geq$ 7.64	52	24			23	53		
< 7.64	29	31	5.62	0.018	35	25	10.80	0.001
APACHE II score								
$\geq$ 17.15	60	25			30	55		
< 17.15	21	30	11.45	0.001	28	23	5.01	0.025
WBC ($\times 10^9$/l)								
$\geq$ 14.37	52	27			28	51		
< 14.37	29	28	3.07	0.080	30	27	4.00	0.046
CRP (mg/l)								
$\geq$ 125	70	27			34	63		
< 125	11	28	22.32	< 0.001	24	15	7.98	0.005
PCT (μg/l)								
$\geq$ 9.51	56	24			28	52		
< 9.51	25	31	8.79	0.003	30	26	4.65	0.031

MAP: mean arterial pressure; SOFA: sequential organ failure assessment; APACHE II: acute physiology and chronic health evaluation II; WBC: white blood cell count; CRP: C-reactive protein; PCT: procalcitonin.

Table 4. Identification of potentially significant predictors for the prognosis of sepsis patients.

Clinical features	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P Value	Hazard ratio	95% CI	P Value
LncRNA CRNDE expression						
High *vs.* low	4.47	1.88–10.66	0.001	3.43	1.15–10.26	0.027
miR-181a-5p expression						
High *vs.* low	0.28	0.13–0.64	0.003	0.41	0.15-0.95	0.042
Gender						
Female *vs.* male	1.02	0.49–2.10	0.961	1.86	0.77–4.52	0.171
Age (yr)						
$\geq$ 56.04 *vs.* < 56.04	1.08	0.52–2.22	0.839	1.26	0.53–3.02	0.603
MAP score						
$\geq$ 0.34 *vs.* < 0.34	1.60	0.72–3.58	0.253	1.01	0.39–2.64	0.981
SOFA score						
$\geq$ 7.64 *vs.* < 7.64	3.84	1.70–8.68	0.001	2.84	1.09-7.36	0.032
APACHE II score						
$\geq$ 17.15 *vs.* < 17.15	5.78	2.23–15.00	< 0.001	3.73	1.28–10.88	0.016
WBC ($\times 10^9$/l)						
$\geq$ 14.37 *vs.* < 14.37	1.33	0.63–2.79	0.45	1.13	0.46–2.75	0.788
CRP (mg/l)						
$\geq$ 125 *vs.* < 125	1.80	0.77–4.23	0.178	0.59	0.19–1.82	0.356
PCT (μg/l)						
$\geq$ 9.51 *vs.* < 9.51	1.47	0.70–3.11	0.312	1.22	0.50–3.00	0.659

MAP: mean arterial pressure; SOFA: sequential organ failure assessment; APACHE II: acute physiology and chronic health evaluation II; WBC: white blood cell count; CRP: C-reactive protein; PCT: procalcitonin.

SOFA score and APACHE II score were also reflective of poor prognosis of patients with sepsis than lower scores ($P < 0.05$).

Contribution of CRNDE and miR-181a-5p to macrophages' release of cytokines

The expression of CRNDE was down-regulated evidently after transfection of si-CRNDE-1, si-CRNDE-2 and si-CRNDE-3 ($P < 0.05$) (Figure 2a). The si-CRNDE-1 was adopted as the silencing strategy for its superior efficacy in lowering CRNDE expression. Regarding miR-181a-5p, transfection of miR-181a-5p mimic resulted in a higher expression of miR-181a-5p than no treatment ($P < 0.05$) (Figure 2b). In addition, LPS-treated cells generated higher levels of NF-kB ($P < 0.001$), TNF-α ($P < 0.001$), IL-1β ($P < 0.001$) and IL-6 ($P < 0.001$) than NC group (all $P < 0.05$) (Figure 2c). Nevertheless, si-CRNDE-1 (Figure 2d) and miR-181a-5p mimic (Figure 2e) seemed to partly block the effects of LPS on cells' secretion of NF-kB, TNF-α, IL-1β and IL-6 ($P < 0.05$), though the action effect of miR-181a-5p mimic was less pronounced than that of si-CRNDE-1 ($P < 0.05$).

The sponging relationship between CRNDE and miR-181a-5p in macrophages

The luciferase activity of psi-Check2-CRNDE Wt + miR-181a-5p mimic group was significantly reduced when compared with psi-Check2-CRNDE Mut + miR-181a-5p mimic group ($P < 0.05$), which showed no significant distinction from psi-Check2 + miR-181a-5p group in terms of luciferase activity ($P > 0.05$) (Figure 2f). Moreover, depression of CRNDE expression could induce up-regulation of miR-181a-5p expression ($P < 0.05$), yet the expression of CRNDE was unaffected after transfection of miR-181a-5p mimic ($P > 0.05$) (Figure 2g).

Modulation of TLR4 expression by CRNDE and miR-181a-5p

Among the patients included, expression of TLR4 in sepsis patients was about 4.5 fold of that in the control population ($P < 0.05$) (Figure 3a), and TLR4 expression was up-regulated with rise of CRNDE expression ($r_s = 0.350$, 95% CI: 0.193–0.489, $P < 0.001$) and decline of miR-181a-5p expression ($r_s = -0.400$, 95% CI: –0.532 to –0.248, $P < 0.001$) (Figure 3b). In addition, transfection of si-TLR4-1 brought about the lowest expression of TLR4 in comparison to si-TLR4-1, si-TLR4-2 and si-TLR4-3 groups ($P < 0.05$), while expression of TLR4 was promoted significantly within cells treated by psi-Check2-TLR4 ($P < 0.05$)

(Figure 3c). Furthermore, co-transfection of psi-Check2-TLR4 Wt and miR-181a-5p mimic observably lowered the luciferase activity of macrophages in comparison to psi-Check2-TLR4 Mut + miR-181a-5p mimic group and psi-Check2-TLR4 Wt + miR-NC group ($P < 0.05$) (Figure 3d). Transfection of si-CRNDE-1 and miR-181a-5p mimic could both decrease the protein level of TLR4 within macrophages ($P < 0.05$) (Figure 3e and 3f), while si-TLR4-1 and psi-Check2-TLR4 could barely alter the expression of CRNDE and miR-181a-5p within cells ($P > 0.05$) (Figure 3g and 3h).

Mediation of TLR4 in regulating the impacts of CRNDE and miR-181a-5p on macrophages' release of cytokines

The mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 were boosted significantly under treatments of LPS and psi-Check2+LPS, when compared with NC group ($P < 0.05$) (Figure 3i). Moreover, dual treatments of psi-Check2-TLR4 and LPS further improved the expression levels of NF-kB, TNF-α, IL-1β and IL-6 when compared with single treatment of LPS ($P < 0.05$). Of note, additional transfection of psi-Check2-TLR4 (i.e. psi-Check2-TLR4 + si-CRNDE + LPS group) was associated with higher mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 than si-CRNDE + LPS group ($P < 0.05$). And treatment of psi-Check2-TLR4 (i.e. psi-Check2-TLR4 + miR-181a-5p mimic + LPS group) could reverse the effects of miR-181a-5p mimic (i.e. miR-181a-5p mimic + LPS group) on macrophages' secretion of NF-kB, TNF-α, IL-1β and IL-6 ($P < 0.05$).

Discussion

Sepsis is a principal trigger of septic shock and multiple organ dysfunction syndrome, with an alarming growth rate of 1.5–9% annually.[28,29] Despite conspicuous advances in anti-infective treatments, the death toll caused by severe sepsis stands high at 30–50%.[29] Since a superfluous expression of pro-inflammatory cytokines, such as TNF-α, IL-1 and IFN-γ, are direct contributors to runaway inflammation and thereby sepsis onset,[30] identification of biomarkers that prohibit inflammatory responses seems particularly vital to curb sepsis progression.

A number of pathways have been suggested to be sepsis related, including MAPK signaling,[31] tyrosine kinase-signaling and transcriptional activator signaling (JAK-STAT),[32] phosphatidylinositol 3-kinase/serine threonine protein kinase (PI3K/AKT) signaling[33] and glycogen synthase-3 (GSK-3) signaling.[33] Nevertheless, there has been scant evidence that verified the role of
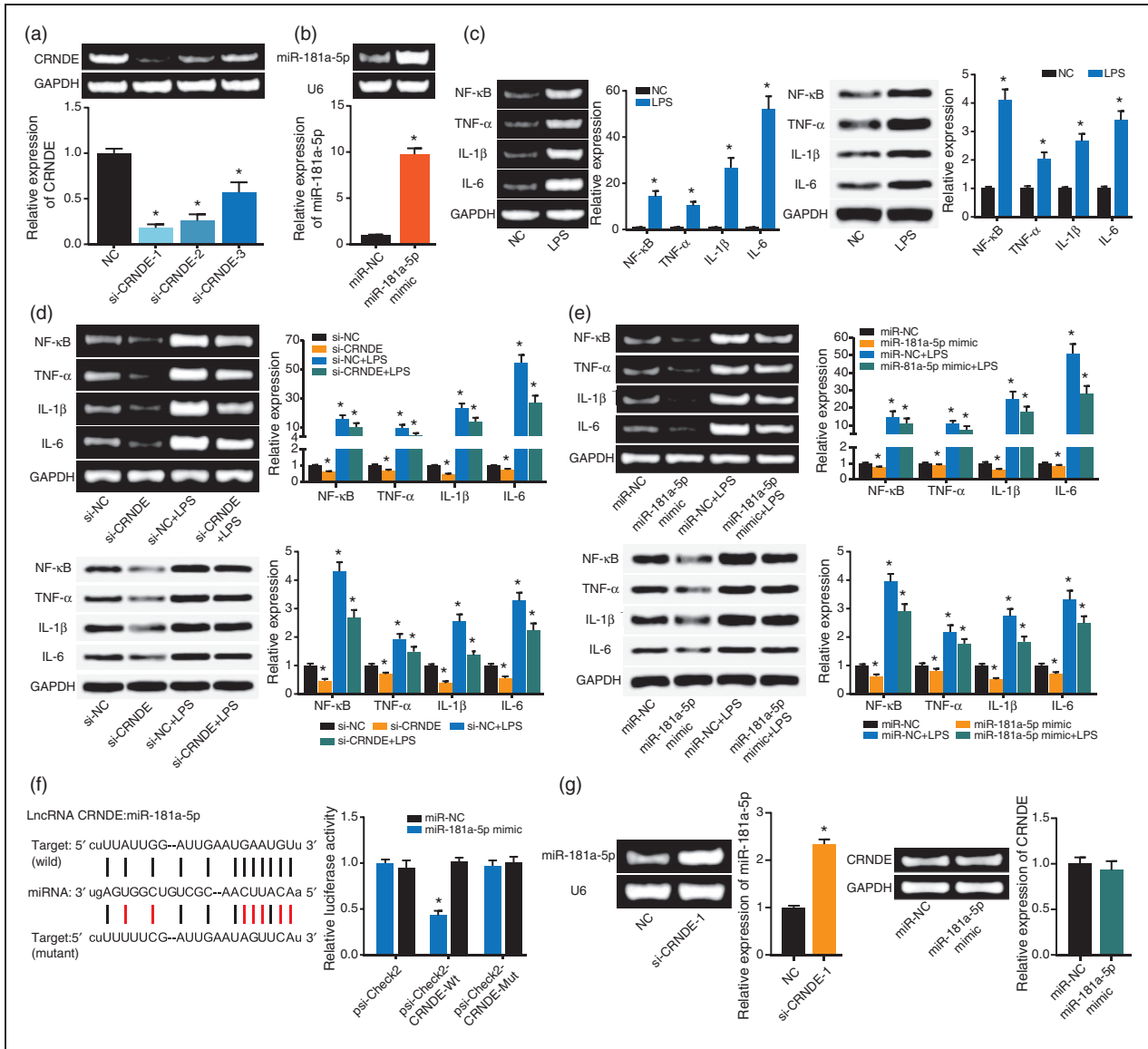
Figure 2. The regulatory effects of CRNDE and miR-181a-5p on the release of cytokines. (a) The expression of CRNDE was determined after respective transfection of si-CRNDE-1, si-CRNDE-2 and si-CRNDE-3. *: $P < 0.05$ when compared with NC group. (b) The expression of miR-181a-5p was measured after transfection of miR-181a-5p mimic. *: $P < 0.05$ when compared with miR-NC group. (c) The mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 within THP-1 cell line were monitored after treatment of LPS. *: $P < 0.05$ when compared with NC group. (d) The mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 within THP-1 cell line were determined among si-NC, si-CRNDE, si-NC + LPS and si-CRNDE + LPS groups. *: $P < 0.05$ when compared with si-NC group. (e) The mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 within THP-1 cell line were compared among miR-NC, miR-181a-5p mimic, miR-NC + LPS and miR-181a-5p mimic + LPS groups. *: $P < 0.05$ when compared with miR-NC group. (f) The CRNDE sponged miR-181a-5p in certain sites, and the luciferase activity was compared among psi-Check2-CRNDE-Wt + miR-181a-5p mimic group, psi-Check2-CRNDE-Mut + miR-181a-5p mimic group and psi-Check2-CRNDE-Wt + miR-NC group. *: $P < 0.05$ when compared with psi-Check2-CRNDE-Wt + miR-NC group. (g) The miR-181a-5p expression was determined after transfection of si-CRNDE-1, and the expression of CRNDE was drawn after treatment of miR-181a-5p. *: $P < 0.05$ when compared with miR-NC group.

lncRNAs in sepsis, although over-expression of lncRNAs HOTAIR and MALAT1 were discovered in septic mouse models.[34,35] The present study investigated lncRNA CRNDE as a potential modulator of inflammatory responses underlying sepsis pathogenesis. Specifically, the clinical analyses demonstrated that CRNDE was differentially expressed within sepsis patients of distinct prognosis (Figure 1c), and
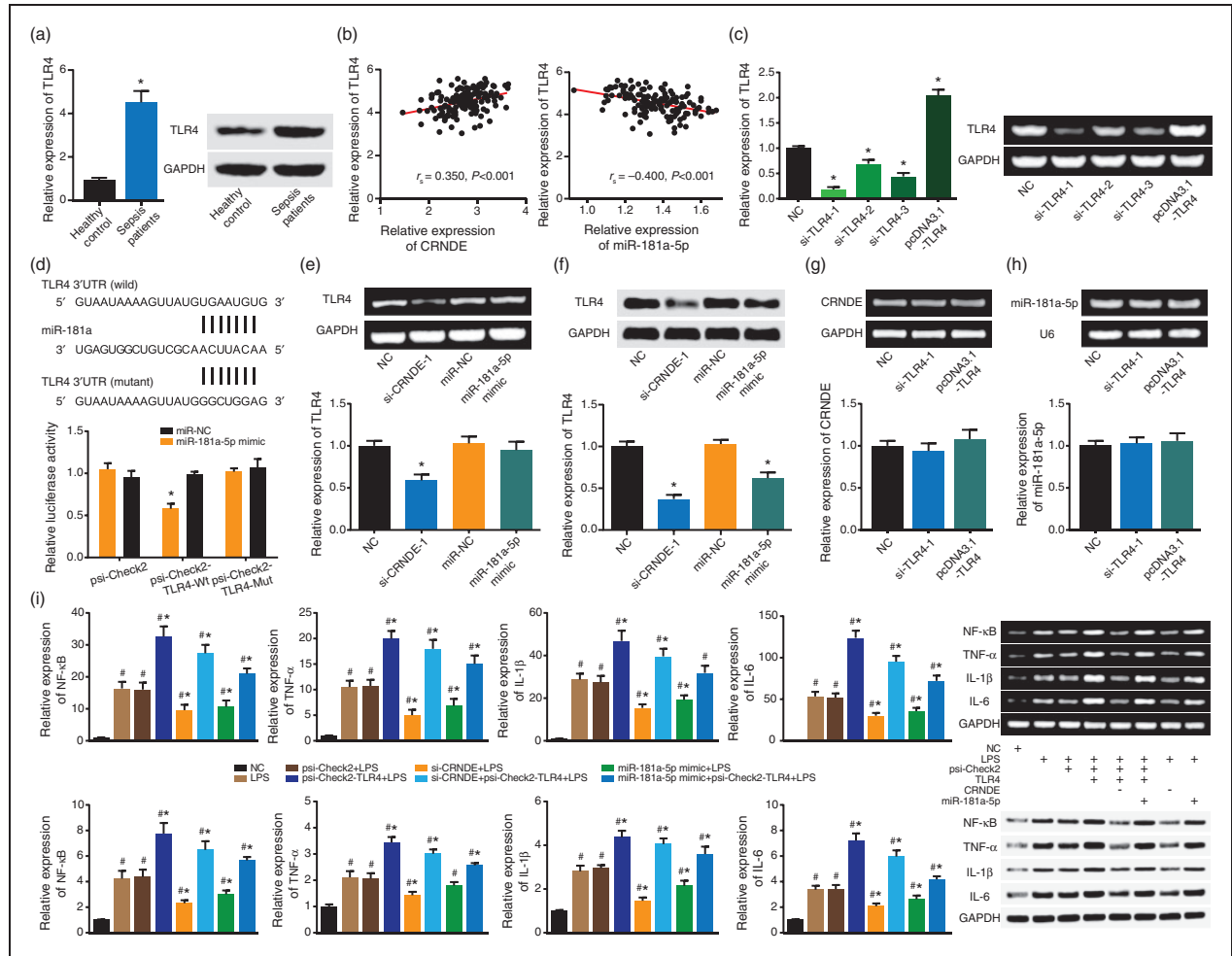
Figure 3. The role of TLR4 in mediating the impacts of CRNDE and miR-181a-5p on the release of cytokines. (a) The TLR4 expression was determined with sepsis patients and healthy controls. *: $P < 0.05$ when compared with healthy controls. (b) The expression of TLR4 assumed a positive correlation with the expression of CRNDE, yet a negative correlation with the expression of miR-181a-5p. (c) The expression of TLR4 was detected after respective treatments of THP-1 cells with si-TLR4-1, si-TLR4-2, si-TLR4-3 and pcDNA3.1-TLR4. *: $P < 0.05$ when compared with NC group. (d) The TLR4 was targeted by miR-181a in certain sites, and the luciferase activity of THP-1 cells was measured among psi-Check2-TLR4-Wt + miR-181a-5p mimic group, psi-Check2-TLR4-Mut + miR-181a-5p mimic group and psi-Check2-TLR4-Wt + miR-NC group. *: $P < 0.05$ when compared with psi-Check2-TLR4-Wt + miR-NC group. (e, f) The expression of TLR4 was assessed under the influence of si-CRNDE-1 and miR-181a-5p mimic. *: $P < 0.05$ when, respectively, compared with NC and miR-NC groups. (g, h) The CRNDE and miR-181a-5p expressions were detected after transfections of si-TLR4-1 and pcDNA3.1-TLR4. *: $P < 0.05$ when compared with NC group. (i) The mRNA and protein levels of NF-kB, TNF-α, IL-1β and IL-6 within THP-1 cell line were determined after respective treatment of NC, LPS, psi-Check2 + LPS, psi-Check2-TLR4 + LPS, si-CRNDE + psi-Check2-TLR4 + LPS, si-CRNDE + LPS, miR-181a-5p mimic + psi-Check2-TLR4 + LPS and miR-181a-5p mimic + LPS groups. #: $P < 0.05$ when compared with NC group. *: $P < 0.05$ when compared with LPS group.

in vitro tests also uncovered that CRNDE could propel expression of sepsis-specific biomarkers, including TNF-α,[36] IL-1β,[37] NF-kB[38] and IL-6[34] (Figure 2). CRNDE had been previously reported as a modulator of sepsis-initiated inflammatory pathways as mentioned above. For instance, the TLR signaling was disordered when CRNDE was over-expressed,[16] and knock-out of CRNDE could dramatically alter the functions of NF-κB signaling, JAK/STAT signaling and PI3K/AKT signaling.[39,40] Consequently, it was

reasonable to speculate CRNDE as a potent biomarker that could greatly influence the inflammatory pathogenesis inherent in sepsis.

In addition, we shed light on the role of miR-181a-5p in CRNDE-caused inflammatory responses, allowing that miR-181a-5p was subjected to targeted modulation of CRNDE (Figure 2f, 2g). In fact, the anti-inflammation function of miR-181a-5p has been documented earlier, such as suppression of NF-κB signaling,[41] down-regulation of IL-8 expression[42] and

blockage of TNF-α expression.[43] Within this study, miR-181a-5p was further demonstrated to impede over-expressed TNF-α, IL-1β, NF-κB and IL-6 that were characteristic for sepsis progression (Figure 2e). Building on these molecular mechanisms, under-expressed miR-181a-5p could serve as a pronounced indicator for poor prognosis of patients with sepsis (Table 3, Figure 1c). Beyond the above, TLR4, whose expression was suppressed under treatments of si-CRNDE and miR-181a-5p mimic (Figure 3d-3f), was discovered to influence the effects of miR-181a-5p on macrophages' secretion of cytokines (Figure 3i). According to the cellular experiments, TLR4-induced inflammation in sepsis cell models was probably mediated by NF-Kb. In fact, there was clear evidence that TLR4 was capable of activating NF-κB,[16] which then entered the nucleus to promote expression of inflammatory cytokines (e.g. TNF-α and IL-1).[44] Intriguingly, the extent to which NF-κB was activated mirrored the prognostic condition of sepsis patients.[45] The linkage of the CRNDE/miR-181a-5p/TLR4 axis and sepsis-related inflammation was thus formed.

Summing up the above, the CRNDE/miR-181a-5p/TLR4 axis was involved in dysfunctional inflammation underlying sepsis onset and development. Nonetheless, a comprehensive understanding of how CRNDE/miR-181a-5p/TLR4 axis plays a part in the underlying sepsis etiology would entail more proof from multiple angles. For instance, CRNDE could sponge more than miR-181a-5p to exert effects on sepsis-centered inflammation, and the miR-181a-5p/TLR4 axis might be influenced by other upstream lncRNAs. As for the clinical part, the included sepsis patients were Chinese-focused, and the derived study results might not be applicable for a sepsis population of other ethnicities. Above all, more convincing evidence is needed to overcome the deficits in experimental design mentioned above.

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Supplemental material

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