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ARTICLE

Associations of cold-inducible RNA-binding protein with bacterial load, proinflammatory cytokines and mortality from pneumonia

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Abstract

Cold-inducible RNA-binding protein (CIRP) is a damage-associated molecular pattern that plays a critical role in triggering inflammatory responses. It remains unknown whether CIRP is strongly associated with bacterial load, inflammatory response, and mortality in sepsis model. Pneumonia was induced in specific pathogen-free 8–9-week old male rats by injecting bacteria via puncture of the tracheal cartilage. The expressions of CIRP and proinflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β] in lung tissues, alveolar macrophages (AMs), plasma, and bronchoalveolar lavage fluid (BALF) were determined by reverse transcription-polymerase chain reaction, western blotting, and enzyme-linked immunosorbent assay. The numbers of bacteria recovered from the lungs were correlated with the bacterial loads injected and mortality. The expressions of CIRP increased sharply as the bacterial loads increased in the lung tissues and AMs. The amounts of TNF-α, IL-6 and IL-1β proteins synthesized were dependent on the bacterial load in the lung tissues. Releases of CIRP, TNF-α, IL-6, and IL-1β increased with the bacterial load in the blood plasma. The proteins confirmed similar patterns in the BALF. CIRP was strongly associated with the releases of TNF-α, IL-6, and IL-1β in the lung tissues, blood plasma, and BALF, and showed a close correlation with mortality. CIRP demonstrated a strong association with bacterial load, which is new evidence, and close correlations with proinflammatory cytokines and mortality of pneumonia in rats, suggesting that it might be an interesting pneumonic biomarker for monitoring host response and predicting mortality, and a promising target for immunotherapy.

Abbreviations: CAP, Community-acquired pneumonia; CIRP, Cold-inducible RNA-binding protein; eCIRP, Extracellular CIRP; DAMP, Damage-associated molecular pattern; IL, Interleukin; BALF, Bronchoalveolar lavage fluid; PBS, Phosphate-buffered saline; AMs, Alveolar macrophages; TNF-α, Tumor necrosis factor-α; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NETs, Neutrophil extracellular traps.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

CIRP is a damage-associated molecular pattern that plays a critical role in triggering inflammatory responses.

WHAT QUESTION DID THIS STUDY ADDRESS?

Our study aimed to evaluate the associations of CIRP with bacterial load, inflammatory response, and mortality in sepsis model.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Expression of CIRP increased sharply as bacterial load increased in pneumonia. CIRP showed close correlations with releases of TNF-α, IL-6 and IL-1β, and mortality of pneumonia.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

CIRP might be an interesting pneumonic biomarker for monitoring host response and predicting mortality, and a promising target for immunotherapy.

INTRODUCTION

Despite substantial advances in treatment, community-acquired pneumonia (CAP) causes high mortality and morbidity worldwide, and is associated with high medical costs.[1,2]

The severity of CAP depends on microbial pathogenicity, load and virulence, and host immunity to infection. Cold-inducible RNA-binding protein (CIRP), identified as a damage-associated molecular pattern (DAMP), plays a critical role in triggering inflammatory responses, and inhibition of CIRP attenuates shock-induced inflammation, tissue injury, and lethality.[3] Increased expression of CIRP was observed in the lungs of mice during sepsis.[4] We previously discovered that CIRP was strongly linked to minor criteria for severe CAP, procalcitonin, interleukin-6 (IL-6), C-reactive protein, sequential organ failure assessment score, pneumonia severity index, and mortality, and best-predicted mortality.[5] Wild-type mice demonstrated significantly more severe sepsis-associated acute lung injury than CIRP knockout mice.[6] However, it remains unknown whether CIRP is strongly associated with bacterial load, inflammatory responses, and mortality in a sepsis model, especially in pneumonia-induced sepsis. Therefore, a study of pneumonia in a rat model was conducted to test this hypothesis.

MATERIALS AND METHODS

Rats

Specific pathogen-free 8–9 week old male Sprague–Dawley rats weighing 200–250 g were purchased from the Experimental Animals Center of Guangdong Province. The rats were housed in temperature-controlled rooms and fed a standard diet. All cages were maintained in the same specific pathogen-free room. All experiments were performed in accordance with the ARRIVE guidelines.

Streptococcus pneumoniae

Pneumonia was induced in rats by infection with *S. pneumoniae* strain ATCC49619. *S. pneumoniae* was plated on blood agar plates overnight and then harvested by centrifugation and resuspended in sterile saline. The final concentration of bacteria was 1.0×10^8 cfu/mL.

Animal ethics declaration

All animal procedures were performed according to the Helsinki convention for the use of animals and approved by the Review Board of Peking University and the Ethical Regulation Committee of Peking University (No. 20220183).

Rat pneumonia models with varied bacterial loads

A total of 128 rats were randomly divided into four groups and the rats in each group were further randomly divided into two subgroups (for sample collection and mortality calculation). There were no cases of exclusion. Rats were anesthetized via an intraperitoneal injection of a solution containing 20% urethan, infected (random injections of

0.2 mL, 0.6 mL and 1.2 mL 10^8 cfu/mL bacteria by a puncture of the tracheal cartilage, respectively. Sterile saline was injected in the normal control group), and sacrificed at the indicated timepoints. Dyspnea, tachypnea, and cyanosis on the third day suggested that the pneumonia model was successful. This study did not include humane end points. The assessors were not blinded. The statistician was blinded to study design (group and subgroup assignment).

Sampling and processing of blood and bronchoalveolar lavage fluid (BALF)

Partial laparotomy and sternotomy were performed, and blood was obtained via direct cardiac puncture on the third day. Blood was centrifuged at 999 g for 10 min to collect plasma. The plasma was frozen at −80°C for further analyses of cytokines and CIRP.

After the blood sampling, the trachea and lungs were exposed. The left main bronchus was clamped and the right lung was lavaged. BALF was obtained by three repeated instillings in and out of the lungs with 3.0 mL of ice-cold sterile 0.9% saline via an endotracheal tube. Next, the BALF was filtered through sterile gauze to exclude mucus plugs and then centrifuged at 999 g for 4 min at 4°C to obtain a cell pellet, and four aliquots of supernatant from each sample were collected and frozen at −80°C until use for further analyses of cytokines and CIRP. The cell pellets were washed with sterilized phosphate-buffered saline (PBS).

Bacterial load quantification

Lungs were dissected into 1 mL Dulbecco's modified eagle medium (DMEM) before homogenizing through a cell strainer. Lung mashes were serially diluted 1:10 in PBS and plated onto blood agar plates. Plates were incubated overnight at 37°C, and individual colonies were counted at appropriate dilutions and averaged to determine CFU per milliliter.

Separation of alveolar macrophages (AMs) from BALF

AMs were isolated from BALF as previously described[7]: cell pellets were resuspended in Roswell Park Memorial Institute (RPMI; Sigma) 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma) and allowed to adhere for a minimum of 3 h in a humidified incubator with 95% air/5% carbon dioxide (v/v) at 37°C. Nonadherent cells were removed by washing with RPMI 1640, leaving adherent AMs, which were >99% pure, as assessed by trypan blue staining and morphological analysis. The AMs were harvested using a cell scraper.

Western blotting

Approximately, 50 mg of tissue samples and the cells frozen at −80°C were lysed in RIPA lysis buffer (Millipore, Massachusetts, USA) supplemented with protease inhibitor (Sigma-Aldrich, St Louis, MO, USA) using TissueLyser II (Qiagen, Hilden, Germany) and on ice for 30 min, respectively. Equal amounts of protein (10 μg) were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Sigma, Burlington, MA, USA), which were subsequently blocked with quick blocking buffer (New Cell & Molecular Biotech, Suzhou, China) for 15 min. Membranes were washed with PBS and then incubated with the following primary antibodies overnight at 4°C: CIRP (1:1000; Proteintech, Wuhan, China), tumor necrosis factor-α (TNF-α) (1:4000; Proteintech, Wuhan, China), IL-6 (1:1000; Sigma-Aldrich), IL-1β (1:3000; Proteintech, Wuhan, China) and β-actin (1:20,000; Proteintech, Wuhan, China), respectively. Horseradish peroxidase-conjugated secondary antibodies were then added and incubated at room temperature for 120 min on a shaking table. After chemiluminescent detection, the Image software was used to analyze the relative amount of each protein band.

Reverse transcription-polymerase chain reaction assay

Total mRNA was extracted from AMs and lung tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed into cDNAs. The polymerase chain reaction was performed at 95°C 3 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final step of 72°C 5 min using the TaqMan Gene Expression Assay (Thermo Fisher Scientific, Rockford, IL, USA) per manufacturer's instructions in a 20 μL reaction to detect the mRNA level of CIRP gene. Gene expression was normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used were as follows: CIRP (GenBank accession no. NM_031147) (forward, 5′-GGG TCC TAC AGA GAC AGC TAC GA-3′; reverse, 5′-CTG GAC GCA GAG GGC TTT TA-3′. The expected band size was 65 bp) and GAPDH (GenBank accession no. X02231) (forward, 5′-GTG CTG AGT ATG TCG TGG AG-3′; reverse, 5′-GTC

TTC TGA GTG GCA GTG AT-3′. The expected band size was 289 bp).

Enzyme-linked immunosorbent assay

The levels of CIRP and proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in blood plasma and BALF were analyzed using commercially available kits (Cloud Clone Corp. Wuhan, China; ThermoFisher, China) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were conducted using Statistical Package for the Social Sciences for Windows version 28.0 (IBM SPSS, Chicago, IL, USA). Continuous variables with normal distributions and categorical variables were described as mean ± standard deviation (SD) and percentages, respectively. Numerical data (CIRP, TNF-α, IL-6 and IL-1β levels) were compared by one-way analysis of variance (ANOVA). Chi-squared test was used for the analyses of categorical variables (mortality). The relationship between variables was estimated by Pearson correlation or Spearman rank correlation. No adjustment for multiplicity was performed for any of the statistical comparisons. Statistical significance was set at p-value <0.05.

RESULTS

Mortality based on bacterial load

The numbers of bacteria recovered from the lungs were correlated with the bacterial loads originally injected by a puncture of the tracheal cartilage (rank correlation coefficient [r_s] value >0.9, $p < 0.001$). There were no deaths in the control group (16 rats). Two of 16 rats died in the 0.2 mL 10^8 cfu/mL bacteria group and 5 of 16 rats in the 0.6 mL 10^8 cfu/mL bacteria group. However, the number of non-survivors increased significantly to 12 of 16 rats in the 1.2 mL 10^8 cfu/mL bacteria group ($x^2 = 24.777$, $p < 0.001$).

Expressions of CIRP in lung tissues and AMs

Interestingly, gene amplification analysis demonstrated that CIRP mRNA expressions increased sharply as the bacterial loads increased in the lung tissues (Figure 1a, $p < 0.001$) and AMs (Figure 1b, $p < 0.001$). The expressions

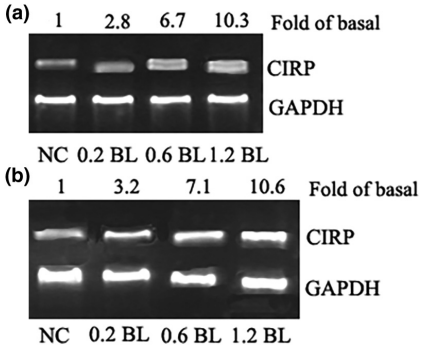

FIGURE 1 CIRP mRNA expression. (a) Gene amplification analysis of CIRP in lung tissues. (b) CIRP mRNA levels in AMs. AMs, Alveolar macrophages; CIRP, Cold-inducible RNA-binding protein; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NC, Normal control. 0.2 BL: 0.2 mL 10^8 cfu/mL bacterial load. 0.6 BL: 0.6 mL 10^8 cfu/mL bacterial load. 1.2 BL: 1.2 mL 10^8 cfu/mL bacterial load. The blots were cropped from same part of the same gel. All p-values <0.001.

of CIRP protein confirmed similar intriguing patterns (all p-values <0.001, Figure 2a for lung tissues and Figure 2b for AMs).

Expressions of TNF-α, IL-6 and IL-1β proteins in lung tissues

The amount of TNF-α protein synthesized was dependent on the bacterial load in the lung tissues, so were IL-6 and IL-1β (all p-values <0.001, Figure 3).

Releases of CIRP, TNF-α, IL-6 and IL-1β proteins in blood plasma and BALF

CIRP release increased with the bacterial load in the blood plasma ($p < 0.001$), and the differences between the groups were significant [all p-values <0.001 and $r_s = 0.968$ ($p < 0.001$)], as did TNF-α, IL-6 and IL-1β proteins (all p-values <0.001, all r_s values >0.9, Table S1 and Figure 4). The proteins confirmed similar patterns in the BALF (all p-values <0.001, all r_s values >0.9, Table S1 and Figure 5).

Associations of CIRP with proinflammatory cytokines and mortality

CIRP was strongly associated with the releases of TNF-α, IL-6 and IL-1β in the lung tissues, blood plasma and BALF (all Pearson correlation coefficients >0.9, all p-values <0.001), and demonstrated a close correlation with mortality ($r_s = 0.586$, $p < 0.001$).

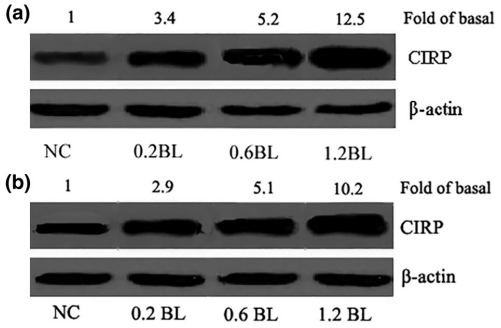

FIGURE 2 Expression of CIRP protein. (a) Western blot analysis of CIRP in lung tissues. (b) Western blot analysis of CIRP in AMs. CIRP, Cold-inducible RNA-binding protein; NC, Normal control. 0.2 BL: 0.2 mL 10^8 cfu/mL bacterial load. 0.6 BL: 0.6 mL 10^8 cfu/mL bacterial load. 1.2 BL: 1.2 mL 10^8 cfu/mL bacterial load. The blots were cropped from different parts of the same gel. All *p*-values <0.001.

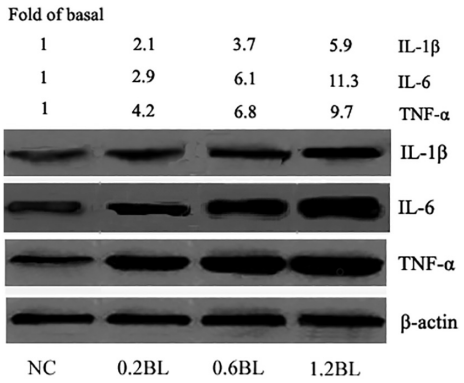

FIGURE 3 Expressions of TNF-α, IL-6, and IL-1β proteins in lung tissues. IL, Interleukin; NC, Normal control; TNF-α, Tumor necrosis factor-α. 0.2 BL: 0.2 mL 10^8 cfu/mL bacterial load. 0.6 BL: 0.6 mL 10^8 cfu/mL bacterial load. 1.2 BL: 1.2 mL 10^8 cfu/mL bacterial load. The grouping of gels/blots was cropped from different gels. All *p*-values <0.001.

DISCUSSION

The numbers of bacteria recovered from the lungs were correlated with the bacterial loads injected and mortality in the current study. CIRP demonstrated a strong association with bacterial load, which is the first known evidence. The amount of TNF-α protein synthesized was associated with the bacterial load in the lung tissues, as did IL-6 and IL-1β. Releases of CIRP, TNF-α, IL-6, and IL-1β increased with the bacterial load in the blood plasma, and the proteins confirmed similar patterns in the BALF. CIRP was strongly associated with the releases of TNF-α, IL-6, and IL-1β in the lung tissues, blood plasma, and BALF, and showed a close correlation with mortality.

CIRP is constitutively expressed at low levels in various tissues and is highly conserved in mammalian cells, and its expression is upregulated under cellular stresses (hypothermic conditions, infection, hypoxia, etc.).[3,8] Regardless of the suppression of global cap-dependent scanning transcription and translation efficiency, CIRP is upregulated partly because of CIRP-mediated transcriptional activation of its own alternative promoters and transcription start sites.[8,9] Upon exposure, CIRP translocates from the nucleus to the cytoplasm through methylation and is stored in stress granules.[10] Different DAMPs share common release mechanisms. The release mechanisms can be largely divided into two categories: passive release mainly due to cell death, and active release from live cells represented by exocytosis (e.g. lysosomal secretion and exosomes).[3,10–12]

Significant and positive correlations between macroscopic lung lesions and *Mycoplasma hyopneumoniae* loads were found.[13] Reduced *Klebsiella pneumoniae* counts in the blood circulation and lungs were correlated with decreased injury and inflammation in the lung lobes and increased survival following combined lung contusion and pneumonia.[14] Similarly, reduced *Pseudomonas aeruginosa* burden in BALF was associated with a reduction in monocyte chemoattractant protein 1 in BALF and serum, chemokine KC in serum, and IL-6 in BALF in a mouse model of acute *Pseudomonas* lung infection.[15] The current study is the first to authenticate a strong association between CIRP and bacterial load. What mechanisms might be envisaged to explore the phenomenon that might imply the pathogenesis of pneumonia? During *Chlamydia pneumoniae* infection, the expression and activity of transcription factor activator protein 1 family proteins c-Jun, c-Fos, and ATF-2 are regulated in a time- and dose (bacterial load)-dependent manner.[16] This may shed a little light on the question, but more studies are warranted to unveil the panorama, to generalize to other species or experimental conditions, and to explore any relevance to human biology.

The plasma level of CIRP was significantly higher in non-survivors than in survivors.[17] We previously found that CIRP was strongly associated with minor criteria for severe CAP.[5] Peak extracellular CIRP (eCIRP) concentrations were higher in patients with severe hypoxemia and independently associated with the degree of respiratory support in COVID-19. The degree of pulmonary involvement measured by computer tomography correlated with eCIRP.[18] Similarly, the expression of CIRP was highly dependent on bacterial load and then was strongly associated with the expressions of proinflammatory cytokines and lethality in the current study, which further corroborates the pivotal role of CIRP in the inflammatory response. What mechanisms might be envisaged to interpret these associations? Recent

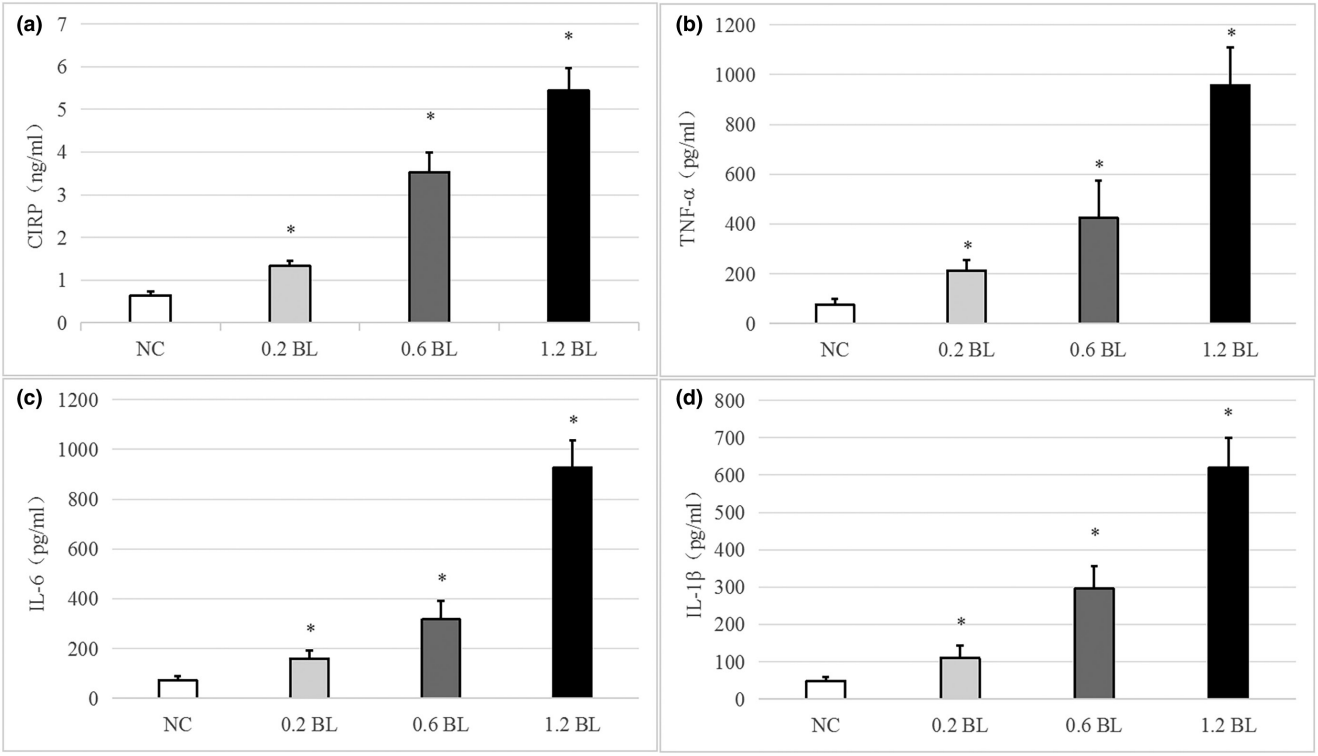

FIGURE 4 (a) Release of CIRP protein in blood plasma. (b) Release of TNF-α protein in blood plasma. (c) Release of IL-6 protein in blood plasma. (d) Release of IL-1β protein in blood plasma. CIRP, Cold-inducible RNA-binding protein; IL, Interleukin; NC, Normal control; TNF-α, Tumor necrosis factor-α. 0.2 BL: 0.2 mL 10^8 cfu/mL bacterial load. 0.6 BL: 0.6 mL 10^8 cfu/mL bacterial load. 1.2 BL: 1.2 mL 10^8 cfu/mL bacterial load. *p-value <0.001 compared with each other.

studies have epitomized that eCIRP can promote acute lung injury via activation of macrophages, neutrophils, pneumocytes, and lung vascular endothelial cells in the context of sepsis, hemorrhagic shock, intestinal ischemia/reperfusion injury, and severe acute pancreatitis.[19] Macrophage erythropoietin receptor signaling is impaired by eCIRP through Rab26 during acute lung injury/acute respiratory distress syndrome, leading to restrained M2 macrophage polarization and delayed resolution of inflammation.[20] Furthermore, eCIRP dysregulates macrophage bacterial phagocytosis during sepsis.[21] Intravenous injection of recombinant murine CIRP in C57BL/6 mice causes lung injury, as evidenced by vascular leakage, edema, increased leukocyte infiltration, and cytokine production in the lung tissue, accompanied by endothelial cell activation and pyroptosis in isolated primary mouse lung vascular endothelial cells.[22] The expressions of CIRP increased with the bacterial loads in the lung tissues and AMs in the current study, which is consistent with the above-mentioned studies. eCIRP induces ferroptosis in septic lungs by decreasing glutathione peroxidase 4 and increasing lipid reactive oxygen species.[23] eCIRP led to increased neutrophil extracellular traps (NETs) formation via Rho activation to exaggerate inflammation in neutrophils in vitro.[24] CIRP induces NETs formation in the lungs during sepsis.[25] Although NETs contribute to pathogen clearance, excessive NETs formation promotes inflammation and tissue damage in sepsis.[26] What mechanisms might be envisaged to elucidate the detrimental activity of NETs? Neutrophil elastase in NETs significantly inhibits efferocytosis by cleaving the macrophage surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. CIRP−/− septic mice exhibited a significantly increased rate of efferocytosis in the peritoneal cavity compared with wild-type mice.[27] eCIRP also induces macrophage extracellular trap formation via activated Gasdermin D, a pore-forming protein involved in NETs formation.[28] Further studies are warranted to reveal the mechanisms underlying the deleterious effects of CIRP as a DAMP in the inflammatory response.

LIMITATIONS

This study has several potential limitations that should be considered. First, as female rats were not included, associations in female rats should be studied. Second, there were relatively small samples in the subgroups used for calculating mortality. Had the numbers been larger, perhaps the results might have been more robust. Finally, the

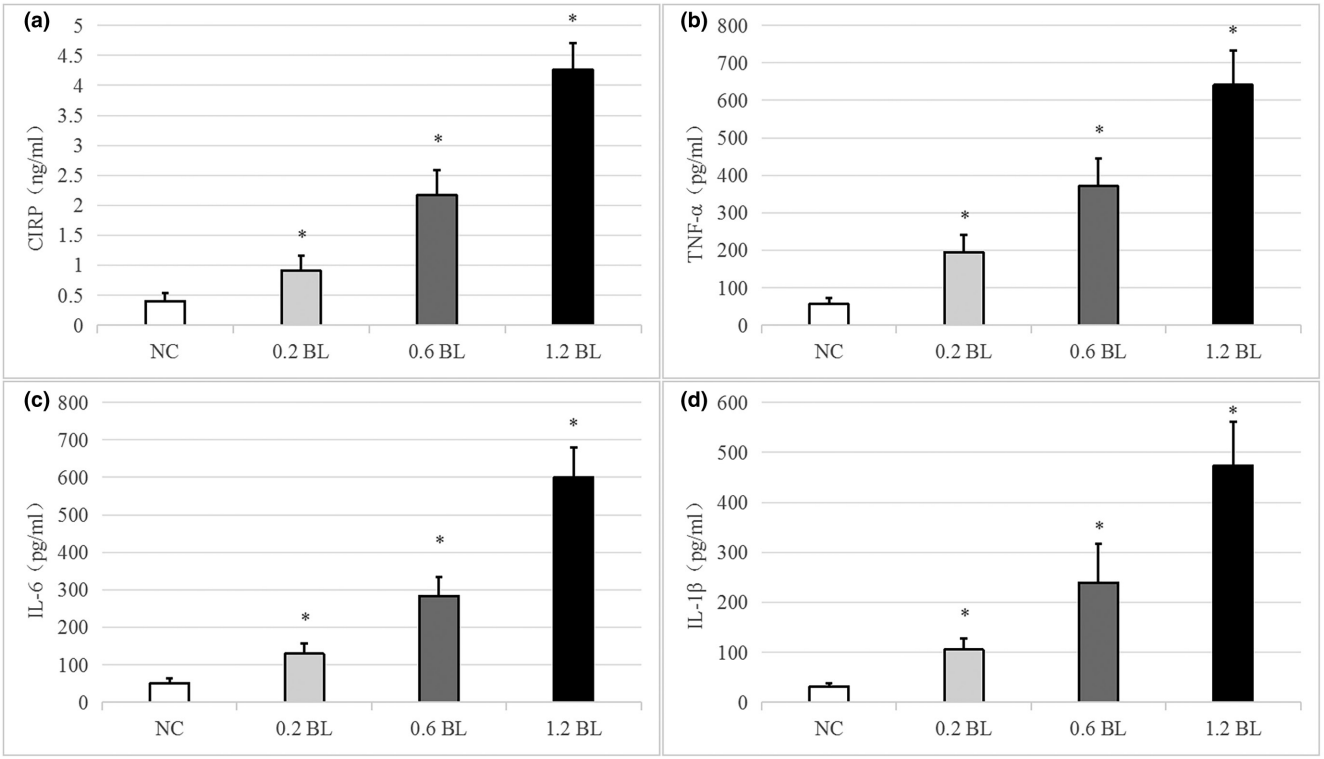

FIGURE 5 (a) Release of CIRP protein in BALF. (b) Release of TNF-α protein in BALF. (c) Release of IL-6 protein in BALF. (d) Release of IL-1β protein in BALF. CIRP, Cold-inducible RNA-binding protein; TNF-α, Tumor necrosis factor-α; IL, Interleukin; NC, Normal control. 0.2 BL: 0.2 mL 10^8 cfu/mL bacterial load. 0.6 BL: 0.6 mL 10^8 cfu/mL bacterial load. 1.2 BL: 1.2 mL 10^8 cfu/mL bacterial load. BALF, Bronchoalveolar lavage fluid. *p-value <0.001 compared with each other.

lack of blinding in the establishment of an animal model is a possible source of bias.

CONCLUSIONS

CIRP demonstrated strong associations with bacterial load, proinflammatory cytokines, and mortality due to pneumonia in rats. Our findings provide new evidence indicating that CIRP might be an interesting pneumonic biomarker for monitoring host response and predicting mortality, and a promising target for immunotherapy.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript; Q.G. and H.Y.L. designed the research; Q.G., H.Y.L., C.Z., and Z.D.L. performed the research; All authors analyzed the data.

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FUNDING INFORMATION

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CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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