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# *Clonorchis sinensis* cyclophilin A immunization protected mice from CLP-induced sepsis

Juan Jiang[a,b,1], Hongling Yin[a,b,1], Yao Sun[c], Huaiqiu Huang[c,*], Xuchu Hu[a,b,**]

[a] Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong, China
[b] Education Ministry Key Laboratory for Tropical Disease Control Research, Sun Yat-sen University, Guangzhou, Guangdong, China
[c] Department of Dermatology and Venereology, the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China

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ABSTRACT

Cyclophilin A (CyPA), ubiquitously existing in cytoplasm of all eukaryotes, can be secreted in response to inflammatory stimuli. Extracellular CyPA plays a prominent role in the pathological processes of inflammatory diseases, acting as a proinflammatory mediator, exerting chemotactic effects, promoting apoptosis of endothelial cells and amplifying ROS generation, thus being considered as a potential treatment target of sepsis, a systemic inflammatory response syndrome. Our previous study found that antibodies against cyclophilin A of *Clonorchis sinensis* (CsCyPA) could neutralize mouse cyclophilin A (MuCyPA). In this study, we explored whether CsCyPA immunization could prevent or ameliorate mice sepsis induced by cecum ligation puncture (CLP). The results showed that CsCyPA immunization could improve the 72 h survival rate of mice after CLP. Moreover, the protective effect presented in a titer-dependent manner. The levels of cytokine IL-1β, IL-6, TNF-α, MCP-1 and AST in serum were remarkably decreased compared to CLP control group mice. Pathological damages of liver, lung and kidney were ameliorated accompanied by less inflammatory cell infiltration. CFU per whole peripheral blood at 12 h and 24 h after CLP surgery was significantly lower than that of CLP control group. In vitro, intracellular ROS generation and cytokine mRNA expression in peritoneal macrophages stimulated by LPS were reduced obviously with anti-CsCyPA antibodies (anti-CsCyPAs) preincubation. All these results demonstrated that CsCyPA immunization protected mice from CLP induced sepsis.

## 1. Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. It remains high mortality in intensive care units, especially in the case of multiple-resistant bacterial infection [2]. New antibiotics development falls behind drug-resistance development, and human will face a drug unavailable circumstance [3,4]. Thus, prevention sepsis instead of prevention infection may be a new way to deal with sepsis caused by various unpredictable objects. The real direct life-threatening elements are the uncontrollable inflammatory responses, severe oxidative stress and circulatory disturbance [5–7]. Targeting to the key pathological factors released by host during infection probably blocks the progress of sepsis. Therefore, identification of the key pathological molecule of host which plays an important role in oxidative stress, inflammatory response and circulatory disturbance is the focus of new strategy against sepsis.

Inspecting pathophysiological process of sepsis, an unattractive protein cyclophilin A interests us as it is an important player taking part in almost all stages of sepsis. Cyclophilin A (CyPA), a highly conversed and ubiquitously expressed cytosolic protein, was first identified as the main target for cyclosporine in 1984 [8–10]. As the most abundant cyclophilin, CyPA is a multifunctional molecule possessing peptidyl prolyl isomerase activity (PPIase activity) and endonuclease activity [11,12]. Intracellularly, it catalyzes the cis-trans isomerization of peptidyl-prolyl bonds of certain proteins and facilitates proteins folding into functional conformation [12]. Besides, CyPA also participates in a range of cell functions, including intracellular trafficking, signal transduction and transcriptional regulation [13,14]. However, CyPA can be secreted into extracellular space spontaneously by various cell types due to inflammatory stimuli, hypoxia, infection, and oxidative stress or released in cell death [15,16]. Secreted CyPA is closely associated with the development and progression of many acute or chronic inflammatory diseases, such as viral infection, periodontitis, and atherosclerosis, tumor, diabetes et al. [17]. During sepsis development,

* Corresponding author.
** Correspondence to: X. Hu, Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong, China.
*E-mail addresses:* hhuaiqiu@medmail.com.cn (H. Huang), huxuchu@mail.sysu.edu.cn (X. Hu).
[1] Co-first author.

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extracellular CyPA acts as a proinflammatory mediator and exerts chemotactic effects on leukocytes via banding with the cell-surface receptor CD147 [18]. Importantly, it has also been demonstrated that CyPA amplified ROS production in positive feedback aggravating oxidative stress, and induced endothelial cell apoptosis leading to circulatory disturbance [19,20].

*Clonorchis sinensis* (*C. sinensis*) is a helminth inhabiting in hepatic biliary ducts of mammal hosts and releases amounts of excretory-secretory proteins (ESPs) to elicit histopathological changes [21,22]. Our previous study found that CsCyPA was a prominent member of CsESPs and could induce high level of anti-CsCyPA antibodies (anti-CsCyPAs) which could cross-react with host CyPA in vitro [23]. We wonder whether the anti-CsCyPAs can neutralize host extracellular CyPA which facilitates sepsis.

Cecal ligation and puncture (CLP) is a golden experimental model for sepsis. In CLP model, large amounts of intestinal bacteria enter abdominal cavity, leading to bacteria peritonitis, followed by bacteria transferring into circulation. This infection activates excessive production of inflammatory factors, chemokines and excessive ROS, causing systemic inflammatory response (SIRS), eventually progressing to multi-organ failure (MOF) and host-defense system collapse [24–27]. In this investigation, we immunized mice with recombinant CsCyPA to produce different titers of antibodies, followed by CLP to evaluate the protection of sepsis by survival rate at 72 h post CLP, pathological changes in tissues, blood bacterial count, cytokines changes, changes of ROS and cytokines mRNA production of peritoneal macrophages.

## 2. Materials and methods

### 2.1. Preparation of recombinant CsCyPA and polyclonal antibodies

Recombinant CsCyPA and anti-CsCyPA antibodies used in this research were produced as previous studies and were endotoxin free [23,28]. The concentration of them was measured by BCA Protein Assay Kit (Thermo, USA) as the manufacturer's instructions.

### 2.2. Animals

All animal experiments in this paper were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of Sun Yat-sen University (Permit Numbers: SCXK (Guangdong) 2016-0029). 180 C57BL/6 male mice (5–6 weeks of age and weighing 19–22 g) were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Animals were allowed to acclimate for 1 week before experiments, housed in a temperature controlled and 12-h light/dark cycle room and allowed access to water, food ad libitum.

### 2.3. In vivo experimental design

First, we assessed the preventive effect of anti-CsCyPAs on CLP induced septic mice through observation of 72 h survival rate. The experiment contained CLP control group, CLP prevention group and Sham group. The CLP prevention group mice were picked out from the mice which were immunized subcutaneously with 50 μg recombinant CsCyPA, followed by one or more boosts of 25 μg antigen to enhance the immune response, producing anti-CsCyPAs. The interval between the first and secondary immunization was 2 weeks and the boosts were at 1-week intervals. 30 mice with anti-CsCyPAs titer of 1:3000/1:10,000/1:20,000 were picked as three CLP prevention subgroups respectively (10 mice each subgroup). Mice of CLP control group (10 mice) were injected with PBS as control.

CLP was performed in three prevention subgroups and control group 10 d after the last injection of CsCyPA or PBS, to induce sepsis. The sham group (10 mice) skipped the steps of cecal perforation, instead the peritoneum was immediately closed after exposure of the cecum. After surgery, all mice received fluid resuscitation via the subcutaneous injection of normal saline (1 ml) and had free access to food and water after recovery from anesthesia. 72 h survival rate was observed to detect the protective effect of anti-CsCyPA antibodies and determined the best antibody titer. Mice which reached the most effective antibody titer after immunization were used as prevention group in the next experiment.

Second, 100 mice (40 CLP control group mice, 40 CLP prevention group mice and 20 Sham group mice), of which CLP prevention mice achieved the most effective antibody titer, were handled with above procedure. Three mice of each group were sacrificed at 6, 12, 24, 48 and 72 h after CLP surgery or sham surgery to collect specimens. Peripheral blood was collected from orbital vein plexus. Serum was separated by centrifugation at 3000*g* for 15 min at 4 °C and then stored at −80 °C. Liver, lung and kidney samples were obtained for histological analysis.

### 2.4. Measurement of cytokines in serum

The concentrations of IL-1β, TNF-α, IL-6, MCP-1, IL-10 and IL-4 in serum were determined by the corresponding enzyme-linked immunosorbent assay kits (CUSABIO BIOTECHCO., Ltd., China) according to the manufacturer's instructions. All samples were measured in triplicate.

### 2.5. Measurement of AST and ALT levels in serum

To estimate the protective function of anti-CsCyPAs on liver injury, we detected CLP-induced elevated levels of AST and ALT in serum. They were determined by common biochemical kits (JianCheng Bioengineering Institute).

### 2.6. Histological analysis

Animal tissue samples were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, sectioned to a thickness of 5 μm and subsequently stained with hematoxylin and eosin. All samples were photographed and examined immediately by Leica DM Microscopes (DM 2500B, Germany, ×200).

### 2.7. Bacteria count

Mouse peripheral blood was collected from orbital vein plexus at 12 or 24 h after CLP. After serial dilutions, 5 μl dilution was coated on blood agar plates. Bacteria were counted after incubation at 37 °C for 24 h and the count was calculated as CFU per whole blood.

### 2.8. Peritoneal macrophage isolation

Primary mouse peritoneal macrophages were harvested from 30 male C57BL/6 mice and isolated as described [29]. The cells were cultured in RPMI 1640 medium with 10% heat-inactivated FCS, 100 U/ml penicillin G and 100 g/ml streptomycin. After 3 h, non-adherent cells were removed and the adherent monolayer cells were used in following in vitro experiments. Cells were maintained at 37 °C in a 5% $CO_2$ air environment.

### 2.9. Cell viability assay

Cell viability was assessed by the Cell Counting Kit. Peritoneal macrophages were seeded into 96-well at a density of $5 \times 10^5$/ml. Cells were treated with anti-CsCyPAs (0–40 μg/ml) and/or LPS (1 μg/ml) for 24 h. Ten μl of CCK-8 solution was added into each well and the cells were incubated at 37 °C for another 2.5 h. The optical density at OD450 nm was determined using a microplate spectrophotometer (Spectra Max; Molecular Devices, Sunnyvale, CA) to determine the cell