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Ethyl pyruvate reverses development of *Pseudomonas aeruginosa* pneumonia during sepsis-induced immunosuppression

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ABSTRACT

Sepsis is characterized by an innate immune response and the following immune dysfunction which can increase the emergence of secondary infections. Ethyl pyruvate (EP) has multiple immunoregulation functions in several serious illnesses, such as burn injury, severe sepsis and acute respiratory syndrome. However, little data was shown the effect of EP administration on immunosuppression post-CLP and the following secondary infection. The cecal ligation and puncture (CLP) followed by the induction of *Pseudomonas aeruginosa* (PA) was used as a clinically relevant two-hit model of sepsis. We assessed the survival rate, lung damage and lung bacterial clearance in vehicle or EP treatment group to demonstrate the lung response to *Pseudomonas aeruginosa* of septic mice. Then cytokines including lung IL-6, IL-1β, IL-10 and plasma HMGB1, apoptosis of splenic immune cells and Foxp3 level on regulatory T cells (Tregs) were studied to demonstrate the mechanisms of EP administration on two-hit mice. We found that the susceptibility of septic mice to Secondary *Pseudomonas aeruginosa* pneumonia could be down-regulated by ethyl pyruvate treatment and the protective effects of EP may via decreasing lung IL-10 and plasma HMGB1 expression, inhibiting the function of Tregs and relieving the apoptosis of splenic immune cells. The “immune paralysis” post-sepsis still remains a rigorous challenge for curing sepsis, our study may aid in the development of new therapeutic strategies to this problem.

1. Introduction

Sepsis is a major cause of mortality among in-hospital patients in all countries [1]. In the past decades, the general theory was that organs of septic patients were impaired by an overwhelming active innate immune response and subsequent effect on endothelial function, blood flow and the metabolism of parenchymal cellular [2]. However, recent studies illustrated that primary infection maybe not the main cause of death in severe septic patients, and secondary infection caused by immunosuppression is more lethal to patients suffered from sepsis [3].

Several studies have shown that T cell exhaustion and an increased percentage of regulatory T cells (Tregs) are vital for sepsis–induced immunosuppression and the following secondary opportunistic infection [2,4,5]. T cells play a critical role in the maintenance of immunity homeostasis, and once the occurrence of T cell-mediated immunosuppression will cause hosts vulnerable to infection [6,7]. An imbalance of Th1 and Th2 cell, especially over-activation of Th2 cells, could be a major cause of the immunosuppression after sepsis [8]. Additionally, a recent review has demonstrated that improvement of T cells function may be a potential immunotherapeutic approach to boost host resistance to infection [9]. Previous study has shown that regulatory T cells is vital in maintenance of immune tolerance and down-regulation of different immune responses [10]. The forkhead/winged helix transcription factor p3 (Foxp3) is a reliable intranuclear marker of Treg subsets [11]. Foxp3 expression and its activity are crucial for the suppressive function of Tregs. Our previous studies have found that the percentage of murine splenic Tregs was significantly increased in 3 days post-sepsis and was likely related to the sepsis-induced immunosuppression. In light of these findings, academics should focus more attention on “paralyzed” status of septic patients, which makes patients susceptible to secondary infections.

Ethyl pyruvate (EP) is a simple ramification derived from the pyruvic acid and has been shown to be an experimental therapeutic on immune dysfunction [12]. Administration of EP could ameliorate organ injury and improve survival rate in animal models of endotoxemia and severe sepsis [13]. One study showed that EP can shift naive T cells to

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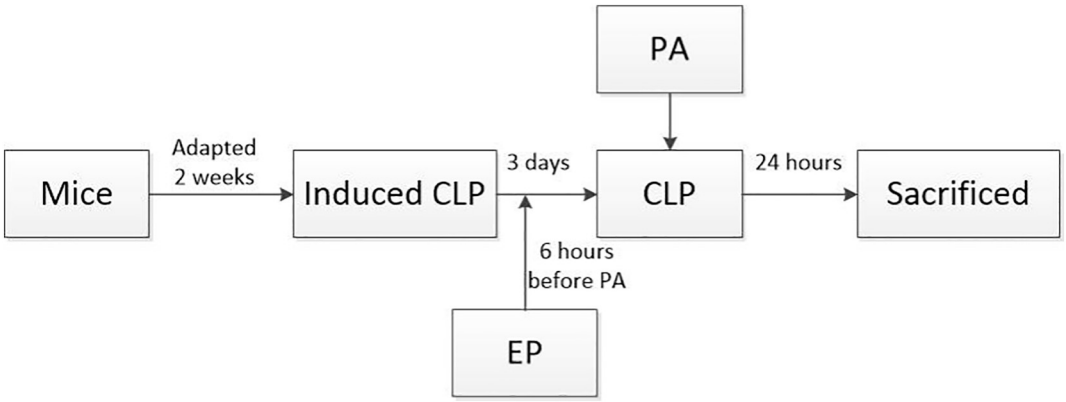

Fig. 1. Schematic diagram of mice underwent cecal ligation and puncture (CLP) after adapting laboratory for two weeks. *Pseudomonas aeruginosa* was administered intratracheally 3 d after CLP. EP-treated mice received EP at 6 h before PA injection. Mice were sacrificed 24 h after PA injection.

Th1 cells in spleen to improve the immune dysfunction of sepsis [8]. In addition, several papers demonstrated that EP could ameliorate the immune dysfunction of murine colitis by down-regulation of HMGB1 release [14,15]. Interaction between released HMGB1 and the receptor for advanced glycation end Products (RAGE) is an important signaling pathway triggering numerous immune dysfunction associated diseases, such as chronic kidney disease and sepsis [16]. Furthermore, previous studies have shown that administration of ethyl pyruvate could relieve the immunosuppression after burn sepsis via activation of T cell or inhibition of Tregs [8]. Thus, we hypothesized that EP could ameliorate the immunosuppression of sepsis and down-regulate susceptibility of septic mice to secondary *Pseudomonas aeruginosa* pneumonia.

2. Materials and methods

2.1. Mice

BALB/c male mice, 6–8 weeks old, were used in our study. All animals were purchased from Shanghai Slack Laboratory Animal Co. Ltd. Animals were raised in the Laboratory Animal Center of Wenzhou Medical University in a temperature- controlled room with 12 h light/12 h dark cyclical alternates, and had free access to water and food. Before experiments, mice were acclimatized in the laboratory for 2 weeks. All experimental manipulations were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Do our best to minimize the number and suffering of the animals used.

2.2. Animal models: cecal ligation and puncture

To induce sepsis, we conducted cecal ligation and puncture model [17]. BALB/c mice were anaesthetized with intraperitoneal injections of amobarbital sodium (0.05 g/kg). After a midline incision (< 1 cm), the distal third of the cecum was ligated and punctured through with a 21-gauge needle. Then a small amount of cecal content was extruded. After the cecum had been returned to the peritoneal cavity, the muscle layer and epidermal layer were sutured. Finally, sterile isotonic saline (24 ml/kg) was administered for volume substitution. Sham-operated mice were undergoing laparotomy with only exposition of cecum without ligation and puncture. Six hours after surgery and then every 12 h until 3 d, mice were intraperitoneal injected with antibiotics at a dose of 25 mg/kg (Imipenem cilastatine, Merck Sharp and Dohme).

2.3. Pseudomonas aeruginosa lung infection

To induce secondary *Pseudomonas aeruginosa* lung infection, mice were anaesthetized with intraperitoneal injections of amobarbital sodium (0.05 g/kg). Then, the trachea was exposed and 20 μl of a bacterial suspension (5×10^5 *Pseudomonas aeruginosa*) was administered intratracheally to sham-operated or CLP mice 24 h before sacrifice [18].

2.4. Experimental design

In the first series of experiments, sepsis was induced by CLP and *Pseudomonas aeruginosa* lung infection was conducted at 3 d post-CLP. We chose the time of 3 d post-CLP to induce secondary infection because previous study has shown the immunosuppression of septic mice was extremely severe at this time [19]. We observed survival rate of each group until the 14th d after conducted secondary infection. Lung bacterial and lung injury was examined 24 h after inducing secondary infection.

In the next series of experiments, ethyl pyruvate (purchased from Sigma-Aldrich) was dissolved in lactated Ringer solution (EP, 28 mM) and administrated to the EP treatment mice at a dose of 40 ml/kg. To determine the effects and mechanisms of ethyl pyruvate on secondary *Pseudomonas aeruginosa* pneumonia post-sepsis in vivo, ethyl pyruvate was administered intraperitoneally to mice 6 h before *Pseudomonas aeruginosa* pneumonia induction. Mice were killed 24 h after inducing secondary infection, and then lung, blood and spleen were harvested to examine histopathology, cytokines or apoptosis (Fig. 1).

2.5. Histological analysis

Lung tissues were dipped in 10% formalin buffer overnight and dehydrated by a graded ethanol series. Tissue samples were embedded in paraffin and cut into 4 μm sections. After stained by haematoxylin and eosin (H & E), lung tissues were examined by light microscopy (magnification × 400). Sections were examined for tissue injury and given a histological score by a pathologist blinded to the treatment groups [20,21]. The score was graded from 0, which represented no consolidation or infiltration, to 5, representing severe infiltration with multiple areas of consolidation, and represented as the mean of 5 fields [22].

2.6. Isolation of splenic Tregs

Mononuclear cells were gathered from spleens of mice. Then, CD4 + CD25 + T-cells were separated from mononuclear cells using magnetic cell sorting (Miltenyi Biotec. Inc.). The procedure referred to the specification. In brief, CD4 + T cells were first gathered by depletion of unwanted cells. Then, CD25 + cells were selected from the CD4 + T cell fraction. Before experiment, the purity of Tregs was ensured more than 90%.