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Anti-sepsis protection of Xuebijing injection is mediated by differential regulation of pro- and anti-inflammatory Th17 and T regulatory cells in a murine model of polymicrobial sepsis☆

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ABSTRACT

Ethnopharmacological relevance: Xuebijing injection (XBJ), a Chinese herbal medicine containing extracts from 5 herbs, is frequently used as an add-on with standard therapies to treat sepsis or septic shock with fewer side effects in China. Nonetheless, its mechanism of action on septic shock remains to be unveiled. We explored the differential effects of XBJ on subtypes of CD4+ T cell differentiation and septic shock protection in a murine model to understand the contribution of XBJ to regulation of the inflammation-immune axis function.

Materials and methods: In vitro T cell differentiation assays were performed to determine the effect of XBJ on CD4+ regulatory T cell and T helper cell differentiation. Besides, 2 ml/kg, 6 ml/kg- and 18 ml/kg of XBJ were administered to different groups of septic mice once/day for 5 days after cecal ligation and puncture (CLP) surgeries. 36 h after CLP, serum levels of pro-inflammatory cytokine TNF-α and IL-6 were determined with Elisa. Frequencies of CD4+ T cells were analyzed after staining with Tregs and T helper cell lineage specific antibodies by flow cytometer.

Results: XBJ at 18 ml/kg stimulated Treg differentiation and moderately inhibited Th17 differentiation *in vitro*. Accordingly, 18 ml/kg XBJ facilitated the expansion of IL-10+ Tregs and normalized pro-inflammatory Th17 population in septic mice. This regimen also significantly reduced serum levels of inflammatory cytokines TNF-α and IL-6 in septic mice. Additionally, 18 ml/kg XBJ injection effectively prevented neutrophil infiltration into the lung and kidney and improved survival in this septic shock model.

Conclusions: In summary, XBJ improves survival in septic shock partially through preventing cytokine storm, inhibiting inflammation and regulating the balance of Tregs and Th17 cells. Thus, higher dose of XBJ is a potential regimen to benefit septic shock patients.

1. Introduction

Sepsis can cause mortality in > 40% of patients when systematic inflammation is out of control (Fink and Warren, 2014). The standard sepsis and septic shock management includes securing the airway, correcting hypoxemia, antibiotics and IV fluid (crystalloids and colloids), nutritional support and Corticosteroids (Dellinger et al., 2013; Sessler et al., 2004) (http://www.uptodate.com).

Cecal ligation and puncture (CLP), the most commonly used preclinical model of sepsis (Hubbard et al., 2005; Rittirsch et al., 2009), reflects clinical reality of polymicrobial sepsis (Anaya and Nathens, 2003). Multi-organ dysfunctional syndrome (MODS), endothelial dysfunction and depletion/inactivation of lymphocytes may contribute to the acute death in this model (Coletta et al., 2014; Hutchins et al.,

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2014). Serum level of IL-6 is a biomarker for survival and disease status in the CLP model (Iskander et al., 2013).

CD4+ T helper 17 cells (Th17) and Tregs play important roles in inflammatory diseases, such as sepsis and rheumatoid arthritis (Noack and Miossec, 2014). Th17 cells represent a pro-inflammatory subset whereas Treg cells promote an anti-inflammatory effect. As important immune regulators, Tregs are characterized by expressing transcription factor Foxp3, which plays a pivotal role in their development, lineage commitment, and regulatory functions (Fontenot et al., 2003; Hori et al., 2003).

Xuebijing injection (XBJ), a Chinese medicine formula combining 5 herbs, is routinely used as an add-on to conventional therapy to treat sepsis and septic shock in China (Jiang et al., 2013; Shi et al., 2016). Combination of XBJ and standard treatment yielded better clinical outcome than standard treatment alone (Gao et al., 2015; Shao et al., 2011). It reduces serum TNF-α concentrations in patients with multiple organ dysfunction syndrome (MODS) (Fang and Wang, 2013), but the mechanism for its attenuation of sepsis remains unclarified.

This study explored the regulatory effects of XBJ on CD4+ T cell differentiation and inflammation in a murine model of septic shock.

2. Materials and methods

2.1. Chemicals and reagents

Xuebijing injection (catalogue number: z20040033, batch number: 1303261) was supplied by Tianjin Chase Sun Pharmaceutical Co., LTD (Tianjin, China). This Chinese medicine is approved by CFDA (China Food and Drug Administration) for treating sepsis and septic shock with CFDA ratification number of GuoYaoZhunZi-Z20040033 for market approval as a drug product. It is routinely used as an add-on to conventional therapy to treat sepsis and septic shock in China (Jiang et al., 2013). This injection contains extracts of 5 herbs, including Carthami Flos (the corolla of Carthamus tinctorius L.), Paeoniae Radix Rubra (the root of Paeonia veitchiiLynch.), Chuanxiong Rhizoma (the root of Ligusticum chuanxiong Hort.), Salviae miltiorrhizae (the root of Salvia miltiorrhiza Bge.) and Angelicae Sinensis Radix (the root of Angelica sinensis (Oliv.) Diels.). Methods of extraction, preparation, and quality control of XBJ were the same as reported previously (Cheng et al., 2016; Huang et al., 2011; Li et al., 2016). Briefly, ingredients from Carthami Flos ("Honghua" in Chinese) were first extracted with ethanol then with water. Ingredients from the other 4 herbs were extracted with water. Finally, XBJ was standardized to contain 1.0–1.7 mg/ml of paeoniflorin and 0.2–0.5 mg/ml of hydroxysafflor yellow A as described (Cheng et al., 2016; Huang et al., 2011; Li et al., 2016).

Anti-Mouse CD28, Anti-mouse IFN-γ, Anti-mouse IL-4, Anti-mouse CD3e, PMA , Ionomycin , Goligistop (Protein Transport Inhibitor) were purchased from BD Biosciences (San Jose, CA), Recombinant Mouse IL-12 and Recombinant IL-4 were purchased from R & D Systems (Minneapolis, MN), Mouse IL-6 ELISA MAXTM standard set and Mouse TNF-α ELISA MAXTM standard set, FITC-anti-mouse CD4, APC rat anti-mouse IL-10, PE-anti-mouse IL-17 were purchased from BioLegend (San Diego, CA).

2.2. Mice

All experiments were performed with weight- (20–25 g) and sex-(male) matched 8–10 week-old C57/B6 mice purchased from Vital River Company (Beijing, China). Mice were acclimated to the standard germ-free housing room under an ambient temperature of 23 ± 2 °C and 40–60% relative humidity, with a diurnal cycle of 12 h light and 12 h dark at the animal facility of Tianjin International Joint Academy of Biotechnology & Medicine for one week before experiments. They were provided with a normal diet and water daily for the entire experiment. All experimental protocols complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996, USA) and the guidelines of Tianjin University of TCM Animal Research Committee (TCM-LAEC2014005). All animal experiments were reviewed and approved by the Committee of Ethics on Animal Experiments at the TJAB (TJAB-JY-2014-0011).

2.3. Septic shock model

A murine sepsis model was recapitulated following the established CLP protocols (Hubbard et al., 2005; Wichterman et al., 1980). In brief, approximately 1 cm midline laparotomy was made on the anterior abdomen to expose the cecum. The cecum was ligated at the point distal to the ileocecal valve and punctured twice with 18G needle. The cecum was gently squeezed to extrude a small amount of fecal content and was left to its original position in the abdominal cavity, and the incision was closed. Sham mice in which the cecum was exposed without ligation and puncture were not subjected to CLP. Chloral hydrate (4%) was delivered via IP injection before the surgery as anesthesia (10 ml/kg body weight); subcutaneous injection of 0.08 mg/kg Buprenorphine was given to mice 30 min before the surgery and every 12 h thereafter for 24 h to prevent the pain. Mice were monitored by trained professionals 2 times/day after the surgery until the end of the experiment. For the survival study, mice were constantly monitored for 5 days. Mice that survived this period of time were euthanized by cervical dislocation.

C57/B6 mice were randomly assigned into seven groups: 1) negative control (n = 5), 2) sham (n = 5), 3) CLP (n = 12), 4) CLP + Dexamethasone (DEX, 1.5 mg/kg, n = 12), 5) CLP + XBJ Low (2 ml/kg, n = 12), 6) CLP + XBJ Medium (6 ml/kg, n = 12), 7) CLP + XBJ High (18 ml/kg, n = 12). Normal saline (NS, 6 ml/kg) were given to the negative control, sham, and CLP groups whereas drugs (DEX or XBJ) were given once daily after CLP surgery. The survival rate was recorded every 24 h for 5 days.

2.4. Ethics statement

The institutional animal ethics committee approved this study design. Given the severity of our study, we diligently observed all mice to minimize suffering within the frames of the experimental design. All mice in the study were housed in the institute's pathogen-free animal facility and the overall health status was checked by trained professionals at least two times per day whenever an animal's condition deteriorated (defined by, among other parameters, decreased activity, progressing hypothermia, rapid weight gain) (Nemzek et al., 2008, 2004). Given that one objective of our study was to determine the effect of XBJ on preventing acute death in septic shock, the time of septic death was a critical endpoint. In survival studies, we followed guidelines recommended by Nemzek et al. to optimize the opportunity for euthanizing moribund mice at the earliest possible time point while assuring the certainty of their imminent mortality to eliminate errors. In detail, mice were euthanized upon signs of impending decease (i.e. inability to maintain upright position/ataxia/tremor and prolonged/deep hypothermia and/or agonal breathing) by cervical dislocation (Nemzek et al., 2008, 2004).

2.5. Cell culture

Splenocytes treated with XBJ *in vivo* (CLP model): Mice from different treatment groups were euthanized at 36 h after the CLP surgery. Spleens were harvested and smashed on a cell strainer. Splenocytes were transferred into a 50-ml tube and centrifuged at (1400 rpm, 7 min, 4 °C). The cells were resuspended in blocking solution (phosphate-buffered saline, 1% normal mouse serum, 0.1% sodium azide) for 10 min, transferred into a fresh 50-ml tube with cell strainer, precipitated, and then resuspended in medium (RPMI1640 containing 0.1% bovine serum albumin, 100 U/ml penicillin-streptomycin, 25 mM HEPES). Cells were counted and distributed into 96-well plates at a