

## Original Article

# Wnt5a/FZD5/CaMKII signaling pathway mediates the effect of BML-111 on inflammatory reactions in sepsis

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**Abstract:** Aims: This study aims to investigate the effect of 5(S), 6(R)-7-trihydroxymethyl heptanoate (BML-111) on the Wnt5a/frizzled-5 (FZD5)/calcium/calmodulin-dependent protein kinase II (CaMKII) signaling pathway in septic mice, and to explore whether this pathway mediates the effect of BML-111 on inflammatory response in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Methods: The cecal ligation and puncture-induced mouse model of sepsis was constructed, and the mice were pretreated with BML-111. In vitro, LPS-induced RAW 264.7 cells were incubated with various concentrations of BML-111. Activation of Wnt5a/FZD5/CaMKII signaling pathway was achieved by transfection of the Wnt5a overexpression plasmid. The levels of interleukin-1 beta (IL-1 $\beta$ ), IL-6 and IL-8 in the mouse serum and cell supernatant were determined by ELISA assay. The expression of Wnt5a, FZD5 and CaMKII $\delta$  was examined by western blot analysis. Results: The results from the in vivo studies revealed that BML-111 shows inhibitory effect on IL-1 $\beta$ , IL-6 and IL-8 expression in the serum of septic mice, and suppresses the expression of Wnt5a, FZD5 and CaMKII $\delta$  protein. The in vitro studies demonstrated that BML-111 inhibits Wnt5a, FZD5 and CaMKII $\delta$  proteins in a dose-dependent manner. BML-111 suppressed the levels of IL-1 $\beta$ , IL-6 and IL-8 in LPS-induced RAW 264.7 cells; however, this effect could be attenuated by transfection of the Wnt5a overexpression plasmid. Conclusion: This study firstly demonstrated that BML-111 suppresses Wnt5a/FZD5/CaMKII signaling pathway in sepsis, and Wnt5a/FZD5/CaMKII signaling pathway mediates the effect of BML-111 on inflammatory reactions. These findings provided a novel molecular basis for the potential effect of BML-111 in sepsis.

**Keywords:** BML-111, sepsis, Wnt5a/FZD5/CaMKII signaling pathway, cytokines

## Introduction

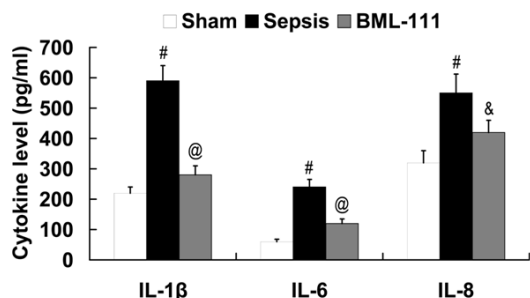
Sepsis is a systemic inflammatory response to infection. It is the leading cause of death in people who have been hospitalized [1, 2], and the incidence of sepsis is about 18 million cases per year worldwide [3].

Lipoxin A4 (LXA4) is a mediator of inflammation derived from arachidonic acid [4]. LXA4 binds to formyl peptide receptor 2/LXA4 receptor (FPR2/ALX) [5, 6], which is a G protein-coupled receptor, and exerts its anti-inflammatory effects. 5(S), 6(R)-7-trihydroxymethyl heptanoate (BML-111) is a synthetic analogue of LXA4. Numerous studies demonstrated that BML-111 involves in inflammation and modulates the immune response [7, 8], and it has been implicated that BML-111 may be a novel therapeutic agent for sepsis [9]. However, the

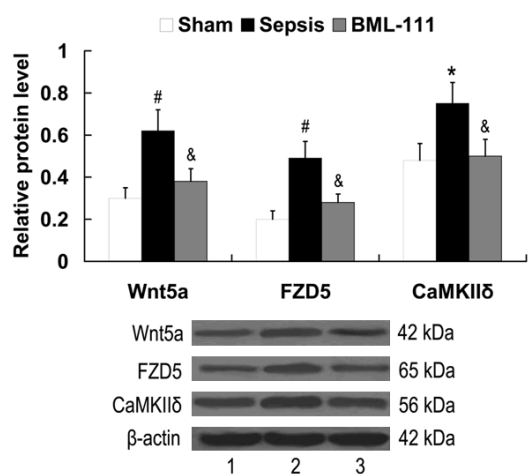
molecular mechanisms underlying the effect of BML-111 on immune response in sepsis is not well understood.

The Wnt pathway is best known for its role in embryogenesis and development [10, 11]. Some researchers highlight that the Wnt pathway is also involved in the regulation of inflammation and immunity [12, 13]. Wnt5a is a member of the highly conserved wingless family. Liu reported that BML-111 inhibits the expression of toll-like receptor (TLR) 2 and TLR4 in the rat intestine following cecal ligation and puncture (CLP)-induced sepsis [9]. In addition, TLR mediated the response of Wnt5a to microbial stimulation [14, 15].

The present study firstly investigated the effect of BML-111 on the Wnt5a/frizzled-5 (FZD5)/calcium/calmodulin-dependent protein kinase



**Figure 1.** Effect of BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression in the serum of mice with sepsis. <sup>#</sup>*P*<0.01 compared with sham; <sup>&</sup>*P*<0.05, <sup>@</sup>*P*<0.01 compared with sepsis. BML-111, 5(S), 6(R)-7-trihydroxymethyl heptanoate; IL, interleukin.



**Figure 2.** Effect of BML-111 on Wnt5a/FZD5/CaMKII signaling in septic mice. Lane 1, sham; lane 2, sepsis; lane 3, BML-111. <sup>#</sup>*P*<0.01, <sup>\*</sup>*P*<0.05 compared with sham; <sup>&</sup>*P*<0.05 compared with sepsis. BML-111, 5(S), 6(R)-7-trihydroxymethyl heptanoate; FZD5, frizzled-5; CaMKII $\delta$ , calcium/calmodulin-dependent protein kinase II delta.

IL (CaMKII) signaling pathway in septic mice, and explored whether this signaling pathway mediates the effect of BML-111 on inflammatory response in lipopolysaccharide (LPS)-induced RAW 264.7 cells.

## Materials and methods

### Experimental model of sepsis

Cecal ligation and puncture (CLP) induced sepsis model was established as described previously [16]. The animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Sichuan Medical University and performed in accordance with the Guide

for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 86-23, revised 1986). The mice were purchased from Shanghai Slac Laboratory Animal Company (Shanghai, China), and anaesthetized by injection of sodium pentobarbital (45 mg/kg) intraperitoneally. Following a mid-line abdominal incision, the cecum was exposed and punctured using the 18G needle (BD, USA). After that, the animals received 1 ml of saline subcutaneously, and were placed in a metabolic cage. Animals in the sham group underwent the same procedure without CLP. Animals in the treatment group received 1 mg/kg BML-111 (Cayman Chemical Company, Ann Arbor, Michigan, USA) intraperitoneally after rehydration. 24 h following CLP, the blood samples were collected and stored at -20°C for centrifugation.

### Cell culture and cell transfection

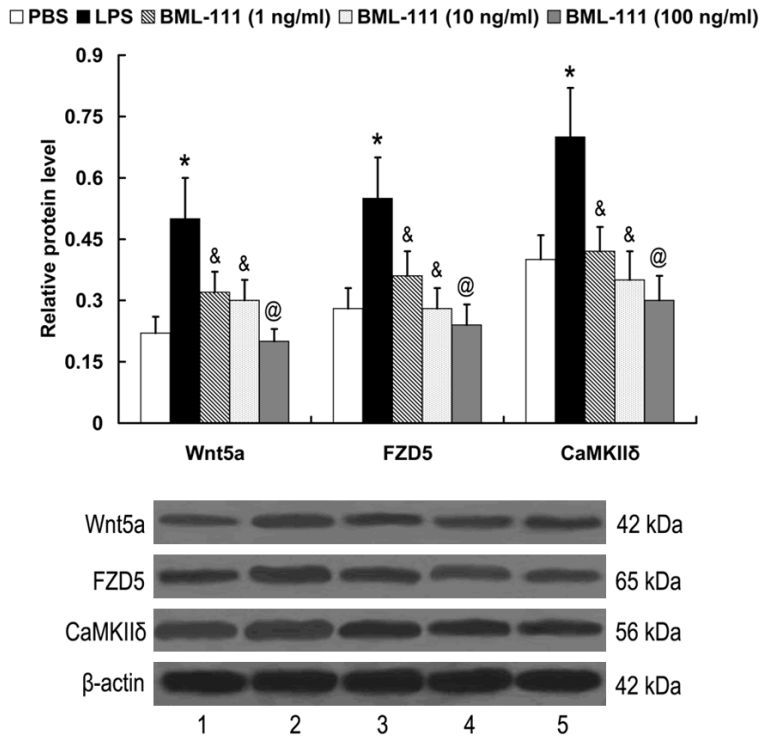
The RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen Life Technologies) at 37°C in 5% CO<sub>2</sub>. LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in phosphate-buffer saline (PBS) to a final concentration of 2  $\mu$ g/ml. Cells were treated with LPS for 12 h. BML-111 was diluted in PBS to the indicated final concentrations. Wnt5a-pcDNA3.1 plasmid was transfected into the RAW 264.7 cells using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

### Measurement of cytokines

ELISA kits for mouse IL-1 $\beta$ , IL-6 were purchased from R&D Systems (Minneapolis, MN, USA), and IL-8 ELISA kit was from Cusabio (Wuhan, Hubei, China). Mouse IL-1 $\beta$ , IL-6 and IL-8 in the serum and culture supernatants were measured by the ELISA kits according to the manufacturer's instructions.

### Western blot analysis

Proteins in the serum and cells were extracted using Total Protein Extraction Kit (KeyGEN BioTECH, Nanjing, Jiangsu, China). Protein samples were separated by SDS-PAGE and were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). All the



**Figure 3.** Effect of BML-111 on Wnt5a, FZD5 and CaMKII $\delta$  expression in LPS-stimulated RAW 264.7 cells. Lane 1, PBS; lane 2, LPS; lane 3, BML-111 (1 ng/ml); lane 4, BML-111 (10 ng/ml); lane 5, BML-111 (100 ng/ml). \* $P < 0.05$  compared with PBS; & $P < 0.05$ , @ $P < 0.01$  compared with LPS. LPS, lipopolysaccharide; BML-111, 5(S), 6(R)-7-trihydroxymethyl heptanoate; FZD5, frizzled-5; CaMKII $\delta$ , calcium/calmodulin-dependent protein kinase II delta.

antibodies used were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). After blocking in 5% non-fat milk, the PVDF membranes were incubated overnight at 4°C with the specific antibodies, including goat polyclonal to Wnt5a (1:800), goat polyclonal to FZD5 (1:400), goat polyclonal to CaMKII $\delta$  (1:800), rabbit polyclonal to  $\beta$ -actin (1:2000), followed by incubation with donkey anti-goat IgG-HRP or goat anti-rabbit IgG-HRP for 2 h at 37°C. A  $\beta$ -actin antibody was used to confirm equal loading. The blots were detected using an ECL western blotting kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Statistical analysis**

All the data were analyzed by SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The statistically significant difference between 2 groups was determined by Student's *t*-test. A *p*-value less than 0.05 was considered significant.

**Results**

*Effect of BML-111 on the expression of IL-1 $\beta$ , IL-6 and IL-8 in the serum of mice with sepsis*

A CLP-induced sepsis model was established to investigate the effect on BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression. Compared with the sham group, the levels of IL-1 $\beta$ , IL-6 and IL-8 were significantly increased in the sepsis group. However, BML-111 showed inhibitory effect on IL-1 $\beta$ , IL-6 and IL-8 expression of mice with sepsis (Figure 1).

*Effect of BML-111 on the expression of Wnt5a, FZD5 and CaMKII $\delta$  in the serum of mice with sepsis*

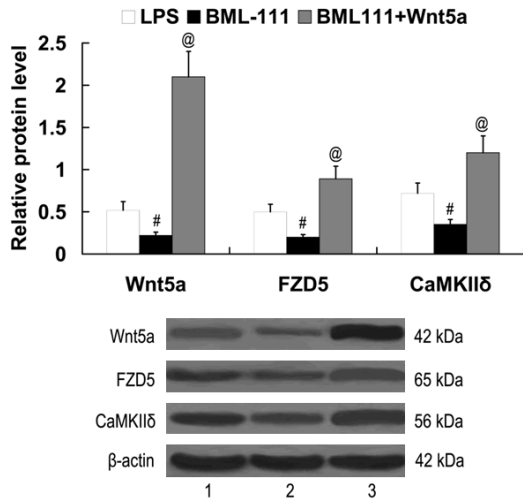
To investigate the effect of BML-111 on Wnt5a/FZD5/CaMKII signaling in septic mice, the expression of Wnt5a, FZD5 and CaMKII $\delta$  in the serum was examined by

western blot analysis. As shown in Figure 2, Wnt5a/FZD5/CaMKII signaling was activated in septic mice, as the relative protein levels of Wnt5a, FZD5 and CaMKII $\delta$  were upregulated in the sepsis group compared with the sham group. Treatment with BML-111 significantly downregulated Wnt5a, FZD5 and CaMKII $\delta$  expression in the serum of mice with sepsis.

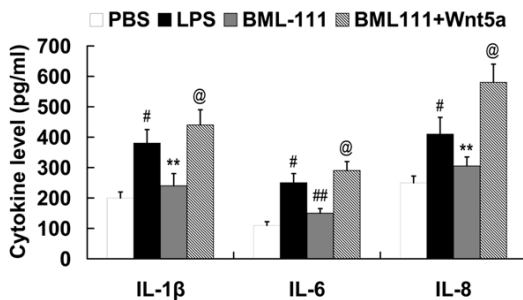
*Effect of BML-111 on the expression of Wnt5a, FZD5 and CaMKII $\delta$  in LPS-stimulated RAW 264.7 cells*

LPS-stimulated RAW 264.7 cells were treated with various concentrations of BML-111 for 24 h, the expression of Wnt5a, FZD5 and CaMKII $\delta$  was then examined by western blot analysis. The results showed that compared with the control, the expression of Wnt5a, FZD5 and CaMKII $\delta$  proteins were induced by LPS. BML-111 inhibited the expression of Wnt5a, FZD5 and CaMKII $\delta$  proteins in a dose-dependent manner in LPS-stimulated RAW 264.7 cells,

## Wnt5a/FZD5/CaMKII signaling pathway mediates the effect of BML-111 on sepsis



**Figure 4.** Expression of Wnt5a, FZD5 and CaMKII $\delta$  in LPS-stimulated RAW 264.7 cells following treatment with BML-111 and transfection of Wnt5a-pcDNA3.1. Lane 1, LPS; lane 2, BML-111; lane 3, BML-111+Wnt5a. # $P$ <0.01 compared with LPS; @ $P$ <0.01 compared with BML-111. LPS, lipopolysaccharide; BML-111, 5(S), 6(R)-7-trihydroxymethyl heptanoate; FZD5, frizzled-5; CaMKII $\delta$ , calcium/calmodulin-dependent protein kinase II delta.



**Figure 5.** Wnt5a/FZD5/CaMKII signaling mediates the effect of BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression in LPS-stimulated RAW 264.7 cells. # $P$ <0.01 compared with PBS; \*\* $P$ <0.01, ## $P$ <0.01 compared with LPS; @ $P$ <0.01 compared with BML-111. LPS, lipopolysaccharide; BML-111, 5(S), 6(R)-7-trihydroxymethyl heptanoate; FZD5, frizzled-5; CaMKII $\delta$ , calcium/calmodulin-dependent protein kinase II delta; IL, interleukin.

with the maximum effect at the concentration of 100 ng/ml (Figure 3).

*Wnt5a/FZD5/CaMKII signaling mediates the effect of BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression in LPS-stimulated RAW 264.7 cells*

To investigate the role of Wnt5a/FZD5/CaMKII signaling in mediating the effect of BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression, Wnt5a-

pcDNA3.1 plasmid was transfected into the LPS-stimulated RAW 264.7 cells, and the cells were treated with 100 ng/ml BML-111 for 24 h. As demonstrated in Figure 4, BML-111 significantly downregulated Wnt5a, FZD5 and CaMKII $\delta$  expression in LPS-stimulated RAW 264.7 cells. However, the expression of Wnt5a, FZD5 and CaMKII $\delta$  proteins was significantly upregulated in the cells transfected with the Wnt5a-pcDNA3.1 plasmid.

As shown in Figure 5, LPS induced the expression of IL-1 $\beta$ , IL-6 and IL-8 in RAW 264.7 cells. Furthermore, we found that the expression of IL-1 $\beta$ , IL-6 and IL-8 was significantly decreased in the LPS-stimulated RAW 264.7 cells by the treatment of BML-111; however, Wnt5a/FZD5/CaMKII signaling activation attenuated the effect of BML-111 on IL-1 $\beta$ , IL-6 and IL-8 expression.

### Discussion

BML-111 has been shown to regulate cytokines in some pathological processes. Wang et al reported that BML-111 shows anti-inflammatory effects on acute pancreatitis-associated lung injury induced by cerulein and LPS in mice. The serum levels of amylase, TNF- $\alpha$ , IL-1 $\beta$  were reduced, and the serum levels of IL-10 were markedly increased by BML-111 pretreatment [17]. In endotoxin exposed rats, BML-111 effectively alleviated morphologic damage of placenta and kidney, serum TNF- $\alpha$  and IL-8 levels, and LPS-reduced proliferation [18]. BML-111 also suppressed airway inflammation of ovalbumin-immunized mice. The levels of IL-1 $\beta$ , IL-4 and IL-8 in serum and bronchoalveolar lavage fluid were suppressed by BML-111 [19]. Recently, Liu et al suggested that BML-111 exerted protective effects on the intestinal mucosal barrier in sepsis by modulating cell apoptosis, upregulating rat defensin-5 mRNA expression levels, as well as regulating the expression of pro-inflammatory and anti-inflammatory cytokine in the intestine, and it may be a novel therapeutic agent for sepsis [9]. In the present study, we examined the expression of cytokines in the serum of mice with sepsis. The results demonstrated that BML-111 also shows anti-inflammatory effects on systemic immune response of mice with sepsis.

IL-1 $\beta$ , IL-6 and IL-8 are important pro-inflammatory cytokines that play vital roles in the patho-

genesis of inflammatory [20-25]. LPS is the principal stimulator of inflammatory reactions [26, 27]. Consistent with the results from our in vivo studies, the in vitro results revealed that BML-111 exerts anti-inflammatory effects in LPS-stimulated RAW 264.7 cells.

Some pathways have been implicated to be related to the action of BML-111, such as nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant responsive element (ARE) signaling pathway, mitogen-activated protein kinase (MAPK)/activator protein-1 signaling pathway [28-30]. Nevertheless, the molecular mechanisms involved in the function of BML-111 in sepsis and LPS-induced inflammatory reactions have not yet been clearly identified. The Wnt pathway plays a distinct role in inflammation and immunity [12, 13]. It has been demonstrated that Wnt5a level is higher in patients with sepsis than in healthy individuals [31]. Wnt5a is induced in infectious and inflammatory diseases, and contributes to innate and adaptive immune responses by ligation to its receptor FZD5, which is independent of the canonical Wnt signaling pathway [32]. Pereira et al suggested that Wnt5a/FZD5/CaMKII signaling pathway was essential for inflammatory macrophage activation [33]. In this study, we investigated the effect of BML-111 on the expression of Wnt5a, FZD5 and CaMKII $\delta$ . Both the in vivo and in vitro studies revealed that BML-111 shows inhibitory effect on Wnt5a/FZD5/CaMKII signaling pathway. Furthermore, the in vitro studies elucidated that Wnt5a/FZD5/CaMKII signaling pathway mediated the effect of BML-111 on inflammatory reactions.

In conclusion, this study firstly demonstrated that BML-111 suppresses Wnt5a/FZD5/CaMKII signaling pathway in sepsis, and Wnt5a/FZD5/CaMKII signaling pathway mediates the effect of BML-111 on inflammatory reactions. This study further expands our knowledge about the action of BML-111 in sepsis and provided a novel mechanism underlying its function in inflammation and immunity.

### Disclosure of conflict of interest

None.

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