International Journal of Biological Macromolecules 88 (2016) 81–87

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

C. butyricum lipoteichoic acid inhibits the inflammatory response and apoptosis in HT-29 cells induced by *S. aureus* lipoteichoic acid

Jinbo Wang[a,*], Lili Qi[a], Lehe Mei[a], Zhige Wu[a], Hengzheng Wang[b]

[a] *Ningbo Institute of Technology, Zhejiang University, Ningbo, Zhejiang 315100, China*
[b] *Centre for Biomolecular Sciences, University of Nottingham, NG7 2RD, United Kingdom*

ARTICLE INFO

Article history:
Received 12 March 2016
Received in revised form 22 March 2016
Accepted 24 March 2016
Available online 26 March 2016

Keywords:
Clostridium butyricum
Staphylococcus aureus
Lipoteichoic acid
Inflammatory response
Apoptosis
HT-29 cells

ABSTRACT

Lipoteichoic acid (LTA) is one of microbe-associated molecular pattern (MAMP) molecules of gram-positive bacteria. In this study, we demonstrated that *Clostridium butyricum* LTA (bLTA) significantly inhibited the inflammatory response and apoptosis induced by *Staphylococcus aureus* LTA (aLTA) in HT-29 cells. aLTA stimulated the inflammatory responses by activating a strong signal transduction cascade through NF-κB and ERK, but bLTA did not activate the signaling pathway. bLTA pretreatment inhibited the activation of the NF-κB and ERK signaling pathway induced by aLTA. The expression and release of cytokines such as IL-8 and TNF-α were also suppressed by bLTA pretreatment. aLTA treatment induced apoptosis in HT-29 cells, but bLTA did not affect the viability of the cells. Further study indicated that bLTA inhibited apoptosis in HT-29 cells induced by aLTA. These results suggest that bLTA may act as an aLTA antagonist and that an antagonistic bLTA may be a useful agent for suppressing the pro-inflammatory activities of gram-positive pathogenic bacteria.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a major foodborne gram-positive bacterium that causes serious food poisoning in humans and animals [1,2]. Food poisoning caused by *S. aureus* accounts for up to 33.3% of bacterial food poisoning cases in the United States [3]. *S. aureus* produces virulence factors such as α-hemolysin, staphylococcal enterotoxins, and toxic shock syndrome toxin-1, which can stimulate severe host cell inflammatory responses [1,4]. Recent studies have reported that lipoteichoic acid (LTA) plays an essential role in the pathogenic processes of *S. aureus* by participating in *S. aureus* adhesion and colonization and stimulating the inflammatory response in host cells [5–7].

LTA is a major cell wall component of gram-positive bacteria and is an amphiphilic molecule composed of polyglycerol phosphate or polyribitol phosphate [8]. Similar to lipopolysaccharides (LPSs) in gram-negative bacteria, LTA is a pathogen-associated molecular pattern (PAMP) molecule in gram-positive pathogenic bacteria and plays essential roles in the bacterial interaction with host cells and in immune response regulation [9]. LTA recognizes Toll-like receptor 2 (TLR2) on the cell membrane. Then, TLR2 activates the NF-κB signaling pathway through MyD88 and induces the expression of downstream inflammatory cytokines (e.g., TNF-α and interleukins [9,10].

LTAs from gram-positive bacteria have substantially different immune-stimulatory effects. For instance, the LTAs from pathogens, non-pathogens, and probiotics have markedly different immune-stimulatory effects on host cells [11]. *S. aureus* LTA (aLTA) can stimulate RAW 264.7 cells to produce and secrete a large amount of inflammatory cytokines. By contrast, *Bacillus subtilis* (*B. subtilis*) LTA has weak immune-stimulatory effects, and *Lactobacillus plantarum* (*L. plantarum*) LTA barely stimulates cells to produce and secrete inflammatory cytokins [11,12]. Additionally, *L. plantarum* LTA can inhibit the inflammatory response stimulated by pathogens and their cellular components or metabolites [13–15]. A mechanistic study suggests that the LTA from *L. plantarum* strain L-137 does not recognize TLR2 or affect the cell inflammatory response; instead, it interacts with scavenger receptor-A (SR-A) on the cell membrane to induce the immune response [16].

Clostridium butyricum (*C. butyricum*) *MIYAIRI* II588 is a gram-positive bacterium isolated from the human intestine; this bacterium can regulate intestinal immune functions and inhibit pathogen-induced inflammatory responses. *C. butyricum MIYAIRI* II588 is used in clinical practice to prevent and treat various intestinal diseases caused by an intestinal flora imbalance [17–19]. Recent studies have proposed that *C. butyricum* is recognized by

* Corresponding author.
E-mail address: wangjb777@126.com (J. Wang).

http://dx.doi.org/10.1016/j.ijbiomac.2016.03.054
0141-8130/

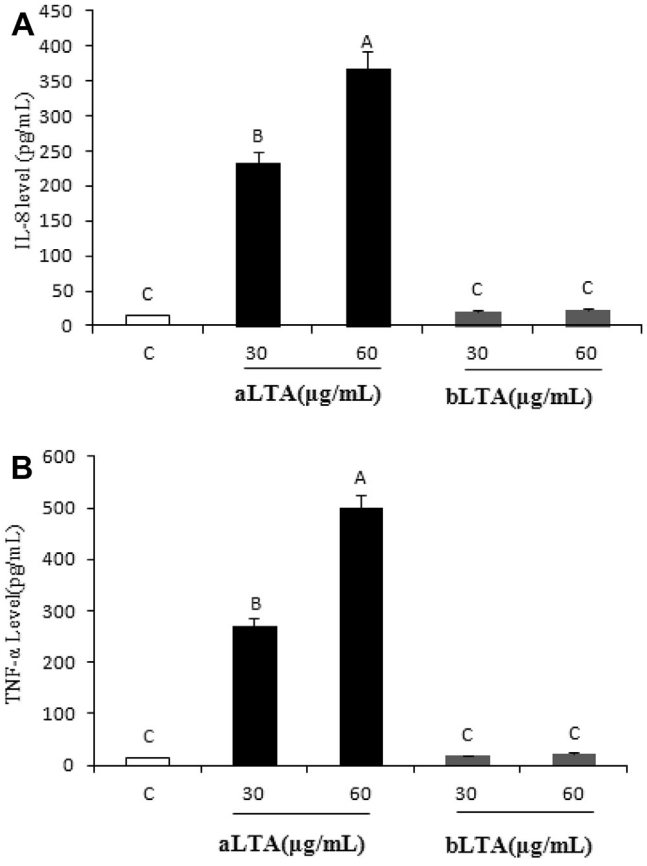

Fig. 1. Effects of aLTA and bLTA on the production of inflammatory cytokines by HT-29 cells. (A) HT-29 cells were treated with the indicated doses of aLTA or bLTA for 12 h and then the concentration of IL-8 in the culture supernatants was determined by ELISA. (B) After treatment with aLTA or bLTA for 12 h, the concentration of TNF-α in the culture supernatants was examined by ELISA.

the TLR2 receptor on immune cell membranes in the intestine and activates the expression and secretion of the anti-inflammatory cytokine IL-10 to inhibit the pathogen-induced intestinal inflammatory response [18,19]. The composition of bacterial components is very complex. Our recent study indicated that *C. butyricum* LTA (bLTA) did not induce inflammatory responses or apoptosis in HT-29 cells, which was obviously different from the effects of aLTA [20]. To clarify whether LTA plays critical roles in the immune-regulatory effects of *C. butyricum*, the effects of bLTA on the inflammatory response and apoptosis in HT-29 cells induced by aLTA was examined in this study.

2. Materials and methods

2.1. Materials

C. butyricum MIYAIRI II588 was obtained from Miyarisan Pharmaceutical Co., Ltd. (Tokyo, Japan). HT-29 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). aLTA was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). DEAE-Sephacel and octyl-Sepharose CL-4B were purchased from GE Healthcare Bio-Sciences Co., Ltd. (Piscataway, NJ, USA). RPMI 1640 medium and fetal bovine serum were purchased from Corning Cellgro Co., Ltd. (Manassas, VA, USA)

The real-time PCR kit was obtained from TaKaRa Co., Ltd. (Dalian, China). The enzyme-linked immunosorbent assay (ELISA) kits were purchased from MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). All other reagents were analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Extraction and purification of LTA

C. butyricum was anaerobically cultured in MRS medium at 37 °C. The overnight bacterial culture was centrifuged for 30 min (4 °C, 5000 rpm), and the cell pellet was stored at −86 °C. LTA was extracted and purified according to the method of Morath et al. [21], with slight modifications. After thawing, 10 g of the cell pellet was disrupted by ultrasonication. The cell lysate was mixed with *n*-butanol at a 1:1 ratio. The mixture was shaken for 30 min to fully release the LTA and then centrifuged for 30 min (4 °C, 5000 rpm) to remove the cell debris. The upper aqueous phase consisting of the crude LTA extract was separated by chromatography using octyl-Sepharose and DEAE-Sephacel sequentially to obtain high-purity LTA. First, hydrophobic chromatography was performed with octyl-Sepharose CL-4B. The aqueous phase was transferred to hydrophobic interaction chromatography (HIC) in 15% *n*-propanol in 0.1 M ammonium acetate, and linear gradient elution was performed using 15–60% *n*-propanol ammonium acetate buffer (pH 4.7, 0.1 M). The LTA peak was collected, and the collected fraction was dialyzed in distilled water for 24 h. The DEAE-Sephacel column was pre-equilibrated with 0.1 M ammonium acetate buffer (pH 4.7). The LTA fraction was loaded onto the DEAE-Sephacel column and eluted with 1.0 M ammonium acetate buffer (pH 4.7). The eluent was collected, and the phosphorus content was determined by a phosphomolybdate-based colorimetric method. The LTA elution peak was collected, and the obtained LTA fraction was dialyzed for 24 h. After dialysis, the LTA fraction was lyophilized to obtain a white LTA powder. Structural characterization and purity determination of the extracted bLTA were performed by 600 MHz nuclear magnetic resonance (^{1}H NMR) and infrared spectroscopy.

2.3. Cell culture

HT-29 cells were cultured at 37 °C in a CO_2 incubator under 5% CO_2. The culture medium was RPMI 1640 containing fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.4. Cell viability assay

HT-29 cell viability was determined using a tetrazolium dye (MTT)-based assay. HT-29 cells were seeded into 96-well plates at a density of 1×10^4 cells/well. Twenty-four hours later, the cells were pretreated with bLTA for 4 h at concentrations of 0, 30, and 60 μg/mL, followed by an incubation with 60 μg/mL of aLTA for 12 h. Then, the cells were gently washed twice with phosphate-buffered saline (PBS). The MTT solution was added to each well at a final concentration of 0.5 mmol/L. The cells were incubated at 37 °C for another 4 h until MTT formazan crystals formed. Then, the medium was aspirated without disturbing the MTT formazan crystals. In total, 200 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the MTT formazan crystals. The absorbance of the resultant solution was measured at 570 nm using a Synergy H1 microplate reader (Biotek, Winooski, VT, USA).

2.5. Hoechst and propidium iodide (PI) staining

HT-29 cells were pretreated with 0, 30, or 60 μg/mL of bLTA for 4 h, followed by 60 μg/mL of aLTA for 12 h. Cell morphology was assessed using the Apoptosis and Necrosis Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, cells were trypsinized and washed three times with PBS. The cells were then stained with Hoechst 33342 (10 ng/mL) and PI (10 ng/mL) for 15 min at 4 °C in the dark.