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Cathelicidin-WA polarizes *E. coli* K88-induced M1 macrophage to M2-like macrophage in RAW264.7 cells

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ABSTRACT

Immune cells - macrophages induced by *E. coli* K88 will lead to a pro-inflammatory response, which is important in host defense. Cathelicidin-WA (CWA) is an efficient antimicrobial peptide (AMP) and can exert immunomodulatory properties. Many studies have demonstrated that AMP can modulate cellular subsets but whether CWA can regulate macrophage polarization by transferring *E. coli* K88-induced M1 macrophage towards M2 one that of anti-inflammation remains unclear. In this study, *E. coli* K88 increased the expression of pro-inflammatory cytokines interleukin-6, interleukin-1β, tumor necrosis factor-α and chemokine CCL3 in RAW264.7 cells with a time-dependent manner, as well as the expression of reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS). On this basis, CWA significantly decreased the pro-inflammatory molecules but increased the anti-inflammatory mediators interleukin-4, interleukin-10 and other M2-related genes in *E. coli* K88-induced macrophages. Western blot analysis indicated that CWA suppressed the expression of TLR-4 and the phosphorylation of STAT1 and NF-κB which modulated M1 macrophage while induced the phosphorylation of STAT6 which activated M2 macrophage. Double staining of M1-specific CD86 and M2-specific CD206 also proved the hypothesis. These results suggested that CWA might dampen the inflammation by modulating M1 phenotype to M2 phenotype in *E. coli* K88-induced macrophages.

1. Introduction

Macrophages are critical immune cells in host defense and inflammation, participating in the handling of infection and keeping homeostasis [1]. In response to different environmental signals, macrophages can display different functional phenotypes including classically activated (M1 or pro-inflammatory) and alternatively activated (M2 or anti-inflammatory) phenotypes [2,3]. M1 macrophage can be generated by toll-like receptor (TLR) ligands such as IFN-γ and/or lipopolysaccharides (LPS), which expresses pro-inflammatory cytokines, reactive oxygen and nitrogen species to exert antimicrobial properties [4–6]. In contrast, stimuli like IL-4 or IL-13 activates M2 macrophage which is considered to serve as the role of dampening inflammatory response, keeping metabolic homeostasis and promoting tissue repair [5,6]. We all know that excessive inflammation can also cause damage, so editing macrophage activation to tuning inflammation by polarizing M1 macrophages to M2 macrophages is of high interest [7].

The molecular mechanisms that determine M1 or M2 polarization involve specific transcription factors as well as posttranscriptional changes. Upon pathogen/damage-associated molecular patterns (PAMPs/DAMPs), signal transducer and activator of transcription 1 (STAT1) and nuclear factor-κB (NF-κB) are two critical transcription factors which result in M1 polarization [8–10]. M1 macrophage expresses pro-inflammatory mediators including IL-1β, IL-6, TNF-α, ROS and chemokines [10,11]. Besides, M1 macrophage specifically regulates the expression of iNOS, inducible enzyme cyclooxygenase-2 (COX-2) and can be marked by CD86 and CD80 [12]. On the other hand, signal transducer and activator of transcription 6/3 (STAT6/STAT3) are key proteins for M2 polarization and the transcription factors up-regulate M2-associated genes such as IL-4, IL-10, transforming growth factor-β (TGF-β), arginase-1 (Arg-1) and mannose receptor CD206 [13–15]. Although many findings have widely proved the stand-alone responses

Abbreviations: CWA, cathelicidin-WA; AMP, antimicrobial peptide; STAT1, signal transducer and activator of transcription 1; p-STAT1, phosphorylated signal transducer and activator of transcription 1; STAT6, signal transducer and activator of transcription 6; p-STAT6, phosphorylated signal transducer and activator of transcription 6; NF-κB, nuclear factor-κB; p-NF-κB, phosphorylated nuclear factor-κB; TLR-4, toll-like receptor-4; IL, interleukin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; COX-2, inducible enzyme cyclooxygenase-2; Arg-1, arginase-1; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; TLR, toll-like receptor; PAMPs/DAMPs, pathogen/damage-associated molecular patterns

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to macrophage, such as LPS or IL-4, more complex responses are observed in the presence of bacterias [16]. As a typical enterotoxigenic *Escherichia coli*, *E. coli* K88 will lead to severe infection diseases but whether it can induce macrophage polarization remains unclear.

Antimicrobial peptides (AMPs) are short cationic molecules that act as a host defense against microbial infection, also called host defense peptides (HDPs) [17]. These peptides not only directly kill microbes but also modulate the immune system of the host [18]. Biochemical and immunological studies indicate AMPs are important in immunomodulation, participating in p38, ERK1/2, NF-κB and other signaling pathways [19,20]. Moreover, some evidences have proved that one consequence of AMPs modulation events is cellular differentiation, which is observed for macrophages and neutrophils [19,21]. For example, when present during monocyte-macrophage differentiation, peptide IDR-1018 induced distinctive macrophage profiles which were intermediate between M1 and M2, with M2-like characteristic and potential M1 immune activation feature [22]. CWA is an effective antimicrobial peptide derived from the endemic genera *Bungarus fascia* [23]. Previous studies have showed that CWA could protect the epithelial barrier and inhibit inflammation in the intestine of both mice and weaned piglets models [24–26]. The aim of our study is to investigate whether CWA can modulate cell polarization in *E. coli* K88-induced pro-inflammatory macrophages.

In this study, we found that *E. coli* K88 induced M1 macrophages with the activation of STAT1/NF-κB signaling pathways and pro-inflammatory mediators. CWA effectively killed all microbes and meantime suppressed the inflammation in *E. coli* K88-induced macrophages. We hypothesised that CWA switched M1 macrophage towards an immunomodulatory phenotype similar to M2 macrophage via STAT6 and other M2-related mediators. In conclusion, our study demonstrated that CWA could dampen the inflammation by regulating macrophage polarization upon *E. coli* infection.

2. Materials and methods

2.1. Materials

CWA was synthesized by GL Biochem (Shanghai, China) and purified at 95.88% as determined by analytical high performance liquid chromatography (HPLC) (Agilent 121 Technologies, CA, USA). CWA was diluted in sterile PBS before use. Standard *Escherichia* strain *E. coli* K88 was purchased from China General Microbiological Culture Collection Center (Beijing, China) and cultured in Luria-Bertani (LB) broth at 37 °C. Primary antibodies specific for β-actin (Abcam, USA), TLR-4 (Abcam, USA), STAT-1 (Epitomics), phosphorylated STAT-1 (Epitomics), STAT-6 (Epitomics), phosphorylated STAT-6 (Epitomics) and phosphorylated NF-κB p65 (Santa Cruz) were purchased.

2.2. Cell culture and E. coli K88 infection

Macrophages (RAW264.7 cell line) were cultured in DMEM medium with high glucose (Yuanpei, China) supplemented with 10% FBS (Gemini, America) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate) at 37 °C with 5% CO_2 in a humidified incubator. For each experiment, 10^6 cells were cultured in 6-well cell culture plates. After 24 h incubation, all plates were washed with PBS to remove the non-adherent cells. The macrophages were treated with DMEM alone (Control) and serial times of 10^6 CFU/ml *E. coli* K88 infection (15 min, 30 min, 45 min and 60 min). After incubation, the cells were washed with cold PBS and collected for RNA or protein extraction.

2.3. CWA treatment to E. coli K88-induced macrophage

Based on the infection model, CWA was subsequently added in *E. coli* K88-induced macrophages for therapeutic. Different concentrations of CWA on cell viability were detected using MTT kit (KeyGEN BioTECH, China) according to the manufacturers' instructions. Macrophages were treated with DMEM alone (Control), 60 min *E. coli* K88 (10^6 CFU/ml), 60 min *E. coli* K88 (10^6 CFU/ml) + 60 min gentamicin (20, 40 μg/ml) and 60 min *E. coli* K88 (10^6 CFU/ml) + 60 min CWA (20, 40 μg/ml). After incubation, cells were washed with precooling PBS and collected for RNA or protein extraction.

2.4. Reactive oxygen species (ROS) detection

Intracellular ROS formation was determined using the fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) (KeyGEN BioTECH, China). Macrophages were treated with serial times of *E. coli* K88 infection (10^6 CFU/ml) and later in the absence or presence of CWA or gentamicin, then macrophages were washed with PBS three times and loaded with 10 μM DCFH-DA for 30 min at 37 °C. After incubation, cells were washed with DMEM medium three times and the level of ROS was detected by confocal laser scanning microscopy (LSM780, ZEISS, Germany).

2.5. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA). The quantity and quality of RNA were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was generated by reverse transcription using 2 μg RNA. Real-time PCR was performed using a StepOne Plus™ system (Applied Biosystems, CA, USA). The primers used for real-time PCR were listed in Table 1. Each reaction included 5 μl Universal SYBR Green Master Mix (Roche, Switzerland), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM) and 4 μl 4-fold diluted cDNA. The thermocycler protocol contained 10 min at 95 °C and 40 cycles of 10s at 95 °C and 35 s at 60 °C, and melt curves were added. 18S was used as housekeeping gene. mRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Western blot

Total protein was extracted with lysis buffer and then quantified by BCA (KeyGEN BioTECH, China). Proteins with different molecular weight were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane. After incubation in 5% skimmed milk powder at room temperature for 1 h, the membranes were incubated in the primary antibodies overnight at 4 °C, followed by incubation in HRP-

Table 1
Primers used for real-time PCR in this study.

Gene	Primer sequence (5′-3′)	Accession number
IL-1β	Forward: TGCCACCTTTTGACAGTGATG Reverse: ATGTGCTGCTGCGAGATTTG	NM_008361.4
IL-6	Forward: TCCTACCCCAATTTCCAATGCT Reverse: TGGTCTTGGTCCTTAGCCAC	NM_031168.2
TNF-α	Forward: TAGCCCACGTCGTAGCAAAC Reverse: TGTCTTTGAGATCCATGCCGT	NM_013693.3
IL-4	Forward: CCAAACGTCCTCACAGCAAC Reverse: AGGCATCGAAAAGCCCGAA	NM_021283.2
IL-10	Forward: AAGGGTTACTTGGGTTGCCA Reverse: CCTGGGGCATCACTTCTACC	NM_010548.2
TGF-β	Forward: GATCACCACAACCCACACCT Reverse: AGGTTCGTGGACCCATTTCC	NM_009368.3
COX-2	Forward: AGCCCATTGAACCTGGACTG Reverse: ACCCAATCAGCGTTTCTCGT	NM_011198.4
Arg-1	Forward: TGCGCCACATGAAAACCATC Reverse: TTGGGAGGAGAAGGCGTTTG	NM_007482.3
CCL3	Forward: TGCGCTGACTCCAAAGAGAC Reverse: CTCGATGTGGCTACTTGGCA	NM_011337.2
iNOS	Forward: GGTGAAGGGACTGAGCTGTTA Reverse: TGAAGAGAAACTTCCAGGGGC	NM_010927.4
18S	Forward: CGAGGGGTTCGGGATTTGTG Reverse: AAAGCCAACCCGAGCGTC	M35283.1