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Treponema pallidum flagellin FlaA2 induces IL-6 secretion in THP-1 cells via the Toll-like receptor 2 signaling pathway

Yafeng Xie[a,b,1], Man Xu[a,1], Yongjian Xiao[a,b], Zhuoran Liu[a,b], Chuanhao Jiang[a], Xingxing Kuang[a], Chuan Wang[a], Haiying Wu[b], Jing Peng[b], Chun Li[b], Yu Wang[b], Huaming Liu[b], Bin Liu[b], Xiaotuan Zhang[b], Feijun Zhao[a], Tiebing Zeng[a], Shuangquan Liu[c], Yimou Wu[a,*]

[a] *Institution of Pathogenic Biology, Medical College, University of South China, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hunan Provincial Key Laboratory for Special Pathogens Prevention and Control, University of South China, Hengyang 421001, China*

[b] *Department of Clinical Laboratory, The Second Affiliated Hospital of University of South China, Hengyang 421001, China*

[c] *Department of Clinical Laboratory, The First Affiliated Hospital of University of South China, Hengyang 421001, China*

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ABSTRACT

Treponema pallidum subsp. *pallidum* membrane proteins are considered as potent inducers in the initiation and development of inflammation. In the present study, the mechanism that leads to the production of interleukin 6 (IL-6), one of the key proinflammatory cytokines, by human monocytic THP-1 cells when these cells are treated with *T. pallidum* flagellin FlaA2 was investigated. Stimulation with flagellin FlaA2 can induce IL-6 expression in human monocytes and augment the phosphorylation of ERK, p38, and NF-κB, but has no effect on the phosphorylation of JNK. Likewise, FlaA2-induced IL-6 production was found to be attenuated by inhibitors for ERK, p38, and NF-κB, but not by JNK inhibitor. Immunofluorescence analysis showed that flagellin FlaA2 could stimulate the translocation of IκBα from the cytosol to the nucleus, and this phenomenon could be inhibited by the specific inhibitor BAY11-7082. FlaA2–induced IL-6 expression was also proved to be abrogated by transfection with dominant negative (DN) plasmid of MyD88. We further demonstrated that transfection with DN-TLR2 was sufficient to attenuate IL-6 expression and the phosphorylation of ERK, p38, and IκBα. These results suggest that flagellin FlaA2 induces IL-6 production via signaling pathways involving TLR2, MyD88, ERK, p38, and NF-κB in monocytes, which could contribute to the pathogenesis of *T. pallidum*.

1. Introduction

Syphilis is a multistage, sexually transmitted illness caused by the obligate human pathogen *Treponema pallidum* subsp. *pallidum* and characterized by protean clinical manifestation. Primary syphilis usually presents as a chancre which contains a high bacterial burgden; secondary syphilis is characterized by a generalized skin rash; it will enter a asymptomatic phage upon resolution. Multiple systems, including cardiovascular system, nervous system, and bone will be involved when progressing to tertiary stage. In contrast to most prokaryotic cell type microorganism, inability to be cultured persistently *in vitro* severely limited the studies on the pathogenesis of *T. pallidum*. The mechanisms involved in eliminating the organism versus those contributing to disease and persistent infection are not yet understood. The main inflammatory response against *T. pallidum* is directed against *T. pallidum* lipoproteins (Liu et al., 2010; Lafond and Lukehart, 2006; Radolf et al., 1995; Salazar et al., 2005).

T. pallidum flagellins, which are very crucial for the motility and chemotaxis of the bacterium (Fraser et al., 1998), includes two flagellar filament outer layer proteins and three flagellar filament core proteins. Tp0664, one of the *T. pallidum* flagellar filament outer layer proteins, is encoded by highly conserved gene *flaA2*. Moreover, computer analyses were performed on flagellin FlaA2 and this protein was predicted to have the potential to contain a thick peptidoglycan layer, which could be recognized by TLR2.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors that are important in the defense against bacterial infection. To date, 13 TLRs have been identified in mammalian animals.

* Corresponding author.
E-mail address: yimouwu@sina.com (Y. Wu).
[1] These authors contributed equally to this work.

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Signal transduction through TLRs activates NF-κB or MAP kinase, and the production of cytokines (Barton and Medzhitov, 2003). Among those TLRs, TLR2, TLR4, TLR5, and TLR6 are well related with the *T. pallidum*. TLR4 recognizes gram-negative lipopolysaccharide (LPS), while TLR2, as a heterodimer with TLR6, mediates immune responses to a broad range of microbial products and is critical for the recognition of bacterial lipopeptides. TLR5 recognizes flagellin (Hayashi et al., 2001), the main component of bacterial flagella which is considered to be critically important for bacterial motility.

IL-6 is a pleiotropic cytokine that has hormone-like attributes and affects the homeostatic process (Hunter and Jones, 2015). Almost all stromal cells and cells of immune system could produce IL-6. In addition, IL-1β and TNF-α are the main activators of IL-6 expression, and a great many pathways, such as TLRs, have been proved to promote the IL-6 secretion. Moreover, IL-6 has been regarded as a better predictor of disease activity than C-reactive protein (Panichi et al., 2004; Fraunberger et al., 2006), which is a sensitive marker of inflammation and infection.

It has been reported that *T. pallidum* lipoproteins could induce inflammatory cytokines secretion through TLR2 signaling pathway, and TLR2 and TLR4 are involved in the innate immune response to the outer sheath components of *B. burgdorferi*. Moreover, a number of studies have revealed that flagellins of many pathogens were recognized by TLR5 and initiated the downstream signal transduction cascade. However, the molecular mechanism of *T. pallidum* flagellins responsible for triggering the inflammatory cascades is still poorly understood. In this study, we investigated whether flagellin Tp0664 could induce inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, in human monocytic cells and further determined the underlying molecular mechanism involved.

2. Materials and methods

2.1. Bacteria and flagellin

T. pallidum Nichols strain was kindly provided by Tianci Yang (Zhongshan Hospital, Medical College of Xiamen University, Xiamen, China), and was grown *in vivo* in adult New Zealand White rabbits as previously described (Lukehart and Marra, 2016; Smajs et al., 2005). Recombinant protein Tp0664 was purified and then processed with Detoxi-Gel Endotoxin Removing Gel column (Thermo Scientific, Waltham, USA). The endotoxin in the recombinant protein was found to be less than 0.04 endotoxin unit (EU)/mL, as assessed by Tachypleus Amebocyte Lysate (Chinese Horseshoe Crab Reagent Manufactory, Ltd., Xiamen, China).

2.2. Cell culture

The human monocytic cell line was purchased from the Center for Type Culture Collection (Wuhan University, Wuhan, China) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini, Woodland, USA), 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. For stimulation experiments, THP-1 cells were seeded in serum-free medium in 6-well plates at a density of 1×10^6/well and then cultivated overnight.

2.3. Transient transfection

Commercially available Smartpool small interfering RNA (siRNA) targeting TLR4 and TLR5, and negative control nontargeting siRNA were obtained from Dharmacon (Thermo Scientific, Waltham, USA). THP-1 cells (1×10^6 cells/well) were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) for 28 h. FuGENE 6 reagent (Roche, Basel, Switzerland) was used for the transfection of the dominant negative (DN)-TLR2, DN-TLR6, and DN-MyD88 according to the manufacturer's instruction. A total of 1×10^6 THP-1 cells were transfected with 0.5 μg DN-TLR2, DN-TLR6, and DN-MyD88. After transfection for 48 h, the cells were incubated with indicated reagents for further experiments.

2.4. Western blotting

Treated and control samples were lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) in the presence of protease inhibitor and phosphatase inhibitor for 60 min in ice-box, and then centrifugated at 4 °C with 10,000 g for 20 min after cells were scraped. Samples were denatured, and equal amounts of protein were subjected to SDS-PAGE, and then transferred to nitrocellulose membrane. The membranes were blocked with 3% bovine serum albumin at room temperature for 1 h and incubated with specific indicated antibodies for 1 h, and then the membranes were incubated with the second antibodies for another 1 h. The immunoreactive bands were detected using an enhanced chemiluminescence Western blotting system G:BOX Chemi XXX9 (Syngene, Cambridge, UK).

2.5. qRT-PCR

Purified RNA (50 ng) was used as template in the quantitative real-time PCR (qRT-PCR) mixture according to the manufacturer's standard protocol for QuantiFast SYBR one-step RT-PCR (Qiagen, Shanghai, China). *IL-6*, *GAPDH*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, and *MyD88* QuantiTect primers were used (Qiagen, Shanghai, China), and quantifications were performed using an LightCycle 96 apparatus (Roche, Basel, Switzerland). The specificity of the PCR was controlled by no-template controls. Results of qRT-PCR are expressed as the fold increase in induction (normalized stimulated cells/normalized unstimulated cells). The percentage of relative gene expression was calculated as the relative amount of MyD88, TLR2, TLR4, TLR5, or TLR6 mRNA in cultures transfected with DN-MyD88, DN-TLR2, TLR4-siRNA, TLR5-siRNA, or DN-TLR6 compared to that of cells transfected with the nontargeting control DN- plasmid or siRNA.

2.6. Cytokine ELISA

Enzyme-linked immunosorbent assay (ELISA) paired antibodies (BD-Pharmingen, San Jose, USA) were used to detect IL-6 secretion in cell-free culture supernatants of THP-1 cells. The ELISA protocol was in accordance with the previous description (Murthy et al., 2000).

2.7. Immunofluorescence staining

THP-1 cells were deposited onto glass coverslips by using a cytocentrifuge (Iris Sample Processing, Inc., Washington, USA), and then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature and permeabilized with 100% MeOH for 10 min. To investigate the cellular localization of p65, cells were treated with a polyclonal antibody against p65 for 2 h. After extensive washing with PBS, cells were incubated with a secondary cy3-conjugated goat-anti-rabbit antibody diluted at 1:1000 in PBS for 1 h at room temperature. Nuclei were stained with 1 mg/mL of 49-6- diamidino-2-phenylindole, and then analyzed by Zeiss LSM 710 Meta microscope (Zeiss, Heidenheim, Germany).

2.8. Statistical analysis

Results are expressed as means ± SEM. Comparison between two groups was determined by Student's *t*-test. Differences were