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Vibrio vulnificus IlpA induces MAPK-mediated cytokine production via TLR1/2 activation in THP-1 cells, a human monocytic cell line

Na Yeon Lee[a], Hye-Yeon Lee[a], Kyu-Ho Lee[b], Seung Hyun Han[c], Soon-Jung Park[a,*]

[a] Department of Environmental Medical Biology and Institute of Tropical Medicine, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea
[b] Department of Environmental Science, Hankuk University of Foreign Studies, Yongin, Republic of Korea
[c] Department of Oral Microbiology and Immunology, Dental Research Institute, and BK21 Program, School of Density, Seoul National University, Seoul 110-749, Republic of Korea

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ABSTRACT

Vibrio vulnificus is a pathogenic bacterium causing primary septicemia, which is followed by a classical septic shock pathway including an overwhelming inflammatory cytokine response. *V. vulnificus* IlpA is a potent immunogenic lipoprotein that triggers cytokine production in human monocytes by activating the toll-like receptor 2 (TLR2). In this study, we further defined the IlpA signaling pathways involved in cytokine production in the human monocytic cell line, THP-1. TLR2 was involved in cytokine production by complexing with TLR1, but not with TLR6. MyD88 was necessary for IlpA-induced cytokine expression through TLR1/TLR2. Three mitogen activated protein kinases (MAPK), p38, ERK1/2, and JNK, were activated in THP-1 cells stimulated with recombinant IlpA (rIlpA). Selective inhibition of each MAPK resulted in significant decrease of rIlpA-induced cytokine production. Especially, functional TLR2 was necessary for IlpA-induced activation of p38 and JNK. IlpA augmented the DNA-binding activity of nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) transcriptional factors to their recognition sites in THP-1 cells. These results suggest that serial activation of TLR1/TLR2, MyD88, the three MAPKs, and NF-κB/AP-1 comprises the signaling pathway responsible for proinflammatory cytokine production by *V. vulnificus* IlpA.

1. Introduction

Vibrio vulnificus is a Gram-negative pathogenic bacterium that is encapsulated, motile, invasive, and found commonly in estuarine environments. It is frequently associated with gastroenteritis, necrotizing wound infections, and primary septicemia, especially in humans with hepatic disease or other immunocompromising conditions (Horeseman and Surani, 2010; Jones and Oliver, 2009). Over 50% of patients with septicemia induced by *V. vulnificus* die of multi-organ failure as a result of a rapidly progressive shock syndrome (Blake et al., 1979; Klontz et al., 1988). Septic shock usually results from the overproduction and dysregulation of host cytokines in response to invading microorganisms. Inflammation-associated cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 (IL-1), play critical roles in the host immune response to microbial infection (Rangel-Frausto, 2005; Salgado et al., 1994). A variety of bacterial products, including lipopolysaccharide (LPS), capsular polysaccharide (CPS), peptidoglycan, lipoarabinomannans, and porins, elicit or modulate cytokine release from host cells both *in vivo* and *in vitro* models (Henderson et al., 1996; Hoshino et al., 1999; Means et al., 1999; Yoshimura et al., 1999). In *V. vulnificus*, CPS directly induces the secretion of proinflammatory cytokines by human peripheral blood mononuclear cells (PBMCs) (Powell et al., 1997).

The initial stage of microbial infection in a host cell is mediated by interactions of proteins on the pathogen surface with host connective tissues or epithelial cells to facilitate bacterial adherence and/or to elicit signal transduction within host cells (Di Martino et al., 2000). Therefore, *V. vulnificus* surface molecules likely interact with host cells, which may trigger cytokine production in immune cells. The immune cells may have specific receptors that interact with molecules on the *V. vulnificus* surface, such as members of the toll-like receptor (TLR) family (Medzhitov et al., 1997; Takeuchi and Akira, 2001). The most abundant outer membrane protein of *V. vulnificus*, OmpU, interacts with host extracellular matrix proteins such as fibronectin (Goo et al., 2006). A screen of the *V. vulnificus*

Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IlpA, immunogenic lipoprotein A; rIlpA, recombinant IlpA; mrIlpA, mutant rIlpA protein; IL, interleukin; TLR, toll-like receptor; HEK 293, human embryonic kidney 293; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BMDMs, bone marrow-derived macrophages; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MyD88, myeloid differentiation primary response protein 88; KO, knockout.

* Corresponding author at: Department of Environmental Medical Biology, Yonsei University School of Medicine, Seoul 120-752, Republic of Korea.
Tel.: +82 2 2228 1843; fax: +82 2 363 8676.
E-mail address: sjpark615@yuhs.ac (S.-J. Park).

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outer membrane proteins for immunogens, led to the isolation of IlpA, a lipoprotein that stimulates production of proinflammatory cytokines via TLR2 in human monocytes (Goo et al., 2007). *V. vulnificus* IlpA is also involved in bacterial adherence to human cell lines, and thus has dual functions as an adhesin and an immunostimulant in *V. vulnificus*-host cell interaction (K.J. Lee et al., 2010).

In this study, we defined the signaling pathways that IlpA, an important virulence factor in *V. vulnificus*, activates in the human monocytic cell line, THP-1, in order to understand its characteristic pathology.

2. Materials and methods

2.1. Cultivation of THP-1 cells

The human monocytic cell line, THP-1 (TIB-202, American Type Culture Collection, Manassas, VA) was cultured in RPMI1640 (Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin (Sigma) at 37 °C in a 5% CO_2 humid atmosphere. For stimulation experiments, cells were seeded at a 2×10^5/mL in 24-well culture plates and incubated in RPMI1640/10% FBS overnight. Cells were then stimulated with rIlpA at varying concentrations and time periods.

2.2. Mice

C57BL/6 mice were obtained from OrientBio (Seoul, Korea) and TLR2 knockout (KO) (TLR2$^{-/-}$), TLR4 KO (TLR4$^{-/-}$), and myeloid differentiation factor 88 (MyD88) KO (MyD88$^{-/-}$) mice were kindly provided by Dr. S. Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan). The animals received humane care in accordance with our institutional guidelines and the legal requirements of Korea.

2.3. Preparation of rIlpA and mrIlpA

The full-length recombinant IlpA (rIlpA) and truncated mutant rIlpA proteins (mrIlpA) were prepared as previously described (Goo et al., 2007). rIlpA was purified to ensure that LPS contamination was <2.5 EU/mL (1 EU = 100 pg) as measured by the Limulus Amebocyte Lysate LPS Detection Kit (Lonza, Basel, Switzerland). Protein samples contaminated with LPS were further purified using the Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL). rIlpA was tested for its ability to induce cytokine production in A498 cells, a CD14-deficient cell line by enzyme-linked immunosorbent assay (ELISA).

2.4. Preparation of bone marrow-derived macrophages and measurement of TNF-α production

Mouse bone marrow-derived macrophages (BMDMs) were differentiated as described by Swanson and Isberg (1995). Briefly, bone marrow cells (BMCs) were obtained by flushing the femora and tibiae with a 26-gauge needle. To generate macrophages, 2×10^6 BMCs suspended in 10 mL Dulbecco's modified Engle's medium (DMEM; Gibco BRL, Karlsruhe, Germany) supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 20 ng/mL recombinant mouse macrophage-colony stimulating factor (mM-CSF; R&D System, Minneapolis, MN) were incubated for 3 days at 30 °C in a humidified 5% CO_2 atmosphere. After removing nonadherent cells, adhered cells were seeded in a 24-well plate (5×10^5 cells per well) containing DMEM/10% FBS with antibiotics and incubated for 2 h. Prepared cells were then challenged with rIlpA, mrIlpA (1 μg/mL), Pam2CGDPKHPKSF (FSL-1), Pam3CysSerLys4 (Pam3CSK4), or LPS (0.1 μg/mL) for 18 h at 37 °C. The supernatants were collected to measure TNF-α secretion using a mouse ELISA kit (BD Biosciences, Franklin Lakes, NJ).

2.5. Cytokine production in TLR1/TLR2 and TLR2/TLR6 cell lines induced by rIlpA

Human embryonic kidney 293 (HEK293) cells stably transfected with either human TLR1/TLR2 or TLR2/TLR6 genes were purchased from InvivoGen (San Diego, CA) and maintained in DMEM supplemented with 4.5 g/L glucose, 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 μg/mL blasticidin S (InvivoGen). For stimulation experiments, stably transfected cells were seeded at a density of 2×10^5 cells in 500 μL of complete medium and allowed to adhere overnight. The cells were then stimulated with mrIlpA, rIlpA (10 μg/mL), FSL-1 (0.1 μg/mL), or Pam3CSK4 (1 μg/mL) for 16 h. Cell-free supernatants were collected, and TNF-α levels were measured using an ELISA kit (BD Biosciences).

2.6. NF-κB reporter assay

HEK293 cells were plated at 1×10^5 cells per well on 24-well culture plates one day before transfection. The seeded cells were transiently transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 50 ng of NF-κB luciferase plasmid [p(IL6κB)$_3$50hu.IL6P-luc+, Vanden Berghe et al., 1998], 5 ng of pRL-TK (Promega, Madison, WI), 0.5 μg of pFLAG-TLR2 (Takashi et al., 2005), and either TLR1 plasmid (InvivoGen) or TLR6 plasmid (InvivoGen). At 24 h after transfection, the cells were stimulated for 6 h with rIlpA (10 μg/mL), FSL-1 (0.1 μg/mL), and Pam3CSK4 (1 μg/mL) without FBS. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.7. Immunofluorescence assay

THP-1 cells stimulated with 40 μg/mL rIlpA for 2 h, were attached to glass slides coated with L-lysine using Cytospin (Thermo, Fremont, CA). The cells were then fixed with chilled methanol (100%) for 10 min, and then treated with acetone for 5 min at −20 °C. After incubating for 1 h in blocking buffer [phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.3), 5% goat serum, and 2% BSA], the cells were incubated overnight with mouse anti-TLR2 (1:50 dilution, BioLegend, San Diego, CA) or mouse anti-TLR1 (1:50 dilution, Abcam Inc., Cambridge, MA) along with rat anti-rIlpA polyclonal antibodies (Abs) (1:100 dilution; Goo et al., 2007) at 4 °C. The cells were subsequently incubated with FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rat IgG (1:200 dilution) (Jackson ImmunoResearch Lab, West Groove, PA) at 37 °C for 1 h. The slides were treated with 1 μg/mL 4′6-diamidino-2-phenylindole (DAPI; Sigma), mounted with anti-fade mounting medium (Vectashield; Vector, Burlingame, CA), and observed under an Axiovert 200 fluorescent microscope (Carl Zeiss, Inc., Oberkochen, Germany).

2.8. Coimmunoprecipitation

THP-1 cells incubated with rIlpA, were disrupted in lysis buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, 1% amidino-phenyl-methyl-sulforyl fluoride, and 1% protease inhibitor), incubated at 4 °C overnight with anti-TLR2 monoclonal Ab (Cell Signaling Technology, Inc., Beverly, MA), and then precipitated with Protein G Sepharose beads. Precipitated proteins were then analyzed by Western blot using anti-TLR2 or anti-MyD88 Abs (Cell Signaling).