Mechanisms of Signal Transduction:

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Vibrio vulnificus IlpA-induced Cytokine Production Is Mediated by Toll-like Receptor 2*

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Sung Young Goo[‡], Yang Soo Han[§], Woo Hyang Kim[‡], Kyu-Ho Lee[§], and Soon-Jung Park[‡1]

From the [‡]Department of Environmental Medical Biology and Institute of Tropical Medicine, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 120-752 and the [§]Department of Environmental Science, Hankuk University of Foreign Studies, Yongin 449-791, Republic of Korea

Vibrio vulnificus* is a pathogenic bacterium causing primary septicemia, which follows a classical septic shock pathway, including an overwhelming inflammatory cytokine response. In this study, we identified a putative lipoprotein of *V. vulnificus*, encoded by the *ilpA* gene, as one of the surface proteins that specifically reacted with the antibodies raised against outer membrane proteins of *V. vulnificus*. Using a mutant *V. vulnificus* in which its *ilpA* gene was knocked out, we found that IlpA is important in the production of interferon-γ in human peripheral blood mononuclear cells. Production of tumor necrosis factor-α and interleukin-6 is also induced by the recombinant IlpA (rIlpA) in human monocytes. Lipidation of the rIlpA was observed by *in vivo* labeling in *Escherichia coli*. Experiments using the mutant IlpA, which is unable to be modified by lipidation, indicate that the lipid moiety of this protein has an essential property for cytokine production in human cells. Pretreatment of monocytes with antibodies against Toll-like receptor 2 (TLR2) inhibited production of both tumor necrosis factor-α and interleukin-6. The role of TLR2 in IlpA-induced cytokine production was confirmed by an *in vitro* assay, in which only the TLR2-expressing cells showed a dramatic induction of nuclear factor-κB activity by rIlpA. In addition, rIlpA treatment resulted in induction of TLR2 transcription in human cells. In comparison with the wild type *V. vulnificus*, the *ilpA* mutant showed a reduced mortality in mice. These results demonstrate that IlpA of *V. vulnificus* functions as an immunostimulant to human cells via TLR2.

Vibrio vulnificus, a Gram-negative bacterium found commonly in the estuarine environment, has been frequently associated with primary septicemia following the consumption of contaminated shellfish. Over 50% of the primary septicemia patients caused by *V. vulnificus* died of multiorgan failure as a result of a rapidly progressive shock syndrome (1, 2).

Extracellular substances produced by *V. vulnificus*, such as hemolytic cytolysin (3, 4) and elastase (5), had been extensively studied as candidate virulence factors responsible for its pathogenesis. Surface structures such as lipopolysaccharide (LPS)[2] (6, 7) and outer membrane proteins (8, 9) were also studied as candidates for *V. vulnificus* virulence factors. Based on the attenuated mouse lethality by a noncapsulated mutant *V. vulnificus*, capsular polysaccharide was also proven to be important in the pathogenesis of *V. vulnificus* (10). Type IV pilin was confirmed to be involved in the virulence of *V. vulnificus* via genetic deletion of the *pilD* or *pilA* genes (11, 12). In addition, motility was discovered as a virulence determinant of *V. vulnificus* (13, 14).

One of the distinct characteristics in *V. vulnificus* pathology is a rapidly progressing septic shock syndrome (15, 16). 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), interleukin-1 (IL-1), and IL-6, play pivotal roles in the host immune response to infection (17, 18). A variety of bacterial products, including LPS, capsular polysaccharide, peptidoglycan, lipoarabinomannans, and porins, has been identified to elicit or modulate the release of cytokines from host cells in both *in vivo* and *in vitro* models (19). For example, initial interaction of *Neisseria gonorrhoeae* with mucosal epithelial cells triggers the release of inflammatory cytokines, including IL-6 and IL-8, which subsequently recruit and activate other immune cells at the site of infection (20, 21).

Therefore, the involvement of *V. vulnificus* surface molecules(s) in its interaction with host cells can be postulated, which may be recognized by immune cells and thus trigger cytokine production. The interaction between the surface molecules of *V. vulnificus* and the immune cells may occur by specific recognition via the receptors on the immune cells. A variety of host cell receptors has been implicated in the recognition of bacteria or their components. One of them is the Toll-like receptor (TLR) family, which plays a central role in innate immune defenses (22, 23). The human TLR family is categorized into at

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ177330.

[1] To whom correspondence should be addressed. Tel.: 82-2-22281843; Fax: 82-2-3638676; E-mail: sjpark615@yuhs.ac.kr.

[2] The abbreviations used are: LPS, lipopolysaccharide; IFN-γ, interferon-γ; PBMCs, peripheral blood mononuclear cells; TNF-α, tumor necrosis factor-α; IlpA, immunogenic lipoprotein A; rIlpA, recombinant IlpA; IL, interleukin; TLR, toll-like receptor; HEK 293, human embryonic kidney 293; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame; PBS, phosphate-buffered saline; OMP, outer membrane protein; F, forward; R, reverse; ELISA, enzyme-linked immunosorbent assay.

TABLE 1

Cell lines, bacterial strains, and plasmids used in this study

The abbreviations used are as follows: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; r, resistant.

Strain/plasmid	Genotype/source	Ref.
Human cell lines		
HEK-293	Hypotriploid human cell line, organ; kidney	ATCC
293-hTLR2/CD14	HEK 293 cells transfected with the plasmids expressing human TLR2 and human CD14	InvivoGen
293-hTLR4/MD2-CD14	HEK 293 cells transfected with the plasmids expressing human TLR4 and human MD2/CD14	InvivoGen
Bacterial strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
BL21 (DE3)	*E. coli* strain B F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal*λ (DE3)	Invitrogen
SM10λ*pir*	*thi thr leu tonA lacy supE recA*::RP4-2-Tc::Mu λ*pir*, *ori*T of RP4, conjugational donor; Km^r	61
XLOLR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 thi-1 recA1 gyrA96 relA1 lac*[*F′ proAB lacI*^{q}ZΔ*M15 Tn10* (Tet^r)] Su^- λ	
V. vulnificus		
ATCC29307	Clinical isolate	ATCC
YS101	Δ*ilpA* mutant of ATCC29307	This study
Plasmids		
$pLAFR_5$	RP4, derivative of pLAFR3 containing a double *cos* cassette; Tc^r	32
pLAFR-ilpA	pLAFR5 with 2,135-bp *V. vulnificus ilpA*; Tc^r	This study
pBK-CMV	Phagemid derived from λZAP® II	Stratagene
pEX3	pBK-CMV, *ilpA*	This study
pBluescript SKII(+)	Cloning vector; *ori*, *oriF1*, *lacZ*; Ap^r	Stratagene
pYS1	pBluescript II SK(+) with 840-bp upstream region of *ilpA*; Ap^r	This study
pYS2	pYS1 with 1,010-bp downstream region of *ilpA*; Ap^r	This study
pDM4	Suicide vector; oriR6K; Cm^r	31
pYS3	pDM4 containing SacI and KpnI fragment of pYS2; Cm^r	This study
pET28b	Expression vector; T7 *lac* promoter, *oriF1*; Km^r	Novagen
pETilpA	pET28 containing 810-bp complete *ilpA* coding region; Km^r	This study
pETmilpA	pET28 containing 741-bp incomplete *ilpA* coding region; Km^r	This study
pFLAG-CMV	N-terminal FLAG expression vector derived from the pCMV5 transient expression vector; CMV promoter, *ori*SV40; Ap^r	Sigma
pFLAG-TLR2	pFLAG-CMV containing 2.3-kb human TLR2 coding region; Ap^r	35
pFLAG-TLR4	pFLAG-CMV containing 2.4-kb human TLR4 coding region; Ap^r	35
$p(IL6\kappa B)_3$50hu.IL6P-luc+	luciferase reporter vector fusing to NF-κB enhancer; *ori*, *oriF1*; Ap^r	36
pCH110	Monitoring and normalizing expression vector in eukaryotic cells; *ori*pBR322, *ori*SV40, *lacZ*; Ap^r	GE Healthcare

least 10 distinct receptors, which convey information in response to different microbial components resulting in activation of nuclear factor-κB (NF-κB), the transcription factor involved in the expression of proinflammatory cytokines, chemokines, and adhesion molecules (24). The ligands recognized by each TLR have the conserved molecular patterns shared by a broad range of pathogens. For instance, TLR4 is a principal signal transducer in the recognition of LPS (25, 26). TLR2 confers responsiveness to various bacterial compounds, such as bacterial lipoprotein (27), lipoarabinomannan (28), lipoteichoic acid (29), and peptidoglycan (30).

In this study, we isolated an outer membrane protein of *V. vulnificus*, which is highly immunoreactive, and identified it as a lipoprotein. The lipidated form of this protein stimulates the production of proinflammatory cytokines in human monocytes. *In vitro* assays further indicate that TLR2 is required for recognition of this *V. vulnificus* lipoprotein.

EXPERIMENTAL PROCEDURES

Cultivation of Bacteria—The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and BL21 (DE3) were grown in Luria-Bertani (LB) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5) supplemented with the appropriate antibiotics at 37 °C. Various strains of *V. vulnificus*, ATCC29307 (wild type), *ilpA* knock-out mutant, and *ilpA* mutant carrying pLAFRilpA were cultured in LBS broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 2% (w/v) NaCl, pH 7.5) at 30 °C with tetracycline (2 μg/ml) when needed.

Immunoscreening of the V. vulnificus Expression Library—Outer membrane proteins (OMPs) were prepared from *V. vulnificus* ATCC29307 and used for intravenous immunization of a rabbit as described (14). The resultant polyclonal antibodies specific to OMPs were used for the immunoscreening experiment. The *V. vulnificus* expression library was constructed by cloning genomic DNA partially digested with Sau3AI into a λZAP®II vector (Stratagene). Recombinant phage DNA was packaged *in vitro* using Gigapack Gold as described by the manufacturer (Stratagene). The amplified library (with a titer of 9×10^6 plaque-forming units/ml) was screened primarily using a 1:2,000 dilution of rabbit antibodies raised against *V. vulnificus* OMPs. Plaques distinct from the background level of antibody binding to the filter were further purified by the second and third screenings. Homogeneous plaques expressing the surface antigens were selected and excised to a pBK-CMV phagemid, as instructed by the manufacturer (Stratagene). The identities of the phagemid inserts were verified by automatic sequencing of the double-stranded plasmids.

Construction of the ilpA Knock-out Mutant V. vulnificus—An 840-bp region upstream of the *ilpA* open reading frame (ORF) was amplified from the genomic DNA of *V. vulnificus* ATCC 29307 using the following two primers: ilpA-upF (5′-GTC-

CGAGCTCGGCGGAGTGAAGTTTGGC-3′; the underlined sequence denotes a SacI restriction site) and ilpA-upR (5′-GCGAGGATCCAGTAAATCTCCTTATTATTTTGAC-3′; the underlined sequence indicates a BamHI restriction site). The PCR product was then cloned into pBluescript SKII (+) to produce pYS1. A DNA fragment containing 1,010 bp downstream of the *ilpA* ORF was generated using the primers, ilpA-downF (5′-GGACGGATCCCAAAGGCGGCGTAGTAAAAG-3′; the underlined sequence denotes a BamHI restriction site) and ilpA-downR (5′-CTGGGGTACCGATTGGGCACTTCTCAGCG-3′; the underlined sequence represents a KpnI restriction site). The DNA fragment was cloned into the corresponding sites of pSY1 resulting in pYS2. An approximate 1.85-kb DNA fragment from pYS2, digested with SacI and KpnI, was ligated with a suicide vector pDM4 (31) to generate pYS3. An *E. coli* SM10 λ*pir* strain carrying pYS3 was conjugated with *V. vulnificus* ATCC29307, and the exconjugants were then selected on a thiosulfate citrate bile sucrose medium supplemented with chloramphenicol. Colonies with characteristics indicating a double homologous recombination event (resistance to 5% sucrose, and sensitivity to chloramphenicol) were further confirmed by PCR using primers ilpA-upF and ilpA-downR, and finally given the name YS101.

*Complementation of Δ*ilpA *Mutant with the Intact ilpA Gene*—An intact *ilpA* gene was amplified from the genomic DNA of wild type *V. vulnificus* ATCC29307 by PCR using the following primer set: ilpA-comF (5′-GGTTGGATCCATTGGTGAGCT-3′; the underlined sequence denotes a BamHI restriction site) and ilpA-comR (5′-TATCAAGCTTCTCTTGGGATCATTTGAAA-3′; the underlined sequence indicates a HindIII restriction site). The amplified *ilpA* DNA fragment of 1,159 bp was digested with BamHI and HindIII, and then cloned into a broad range host plasmid, pLAFR5 (32), resulting in the positioning of *ilpA* ORF downstream of the *lac* promoter in pLAFR5. The resultant plasmid, pLAFRilpA, in *E. coli* SM10 λ*pir* was transferred into the *ilpA* mutant (YS101) by conjugation, and the exconjugants were selected on thiosulfate citrate bile sucrose agar containing tetracycline (2 μg/ml).

Expression and Purification of Recombinant IlpA Proteins—Two oligonucleotides, lipo-F (5′-CATGCCATGGCTATGAAATTTAGCCTTAAAGG-3′; the underlined sequence denotes an NcoI restriction site) and lipo-R (5′-CCCAAGCTTCCAGCCTTTTACTACGCC-3′; the underlined sequence represents a HindIII restriction site), were used to amplify an 810-bp DNA fragment containing the complete ORF of the *ilpA* gene from the genomic DNA of *V. vulnificus*. NcoI and HindIII sites located at both ends of the resultant *ilpA* DNA were used to clone this DNA into the pET28b expression plasmid (Novagen) to generate the plasmid pETilpA. The recombinant IlpA (rIlpA) protein was overexpressed in *E. coli* BL21(DE3) carrying pETilpA by adding isopropyl thio-β-D-galactoside (IPTG; Sigma) at a concentration of 1 mM, and purified using a Ni^{2+}-nitrilotriacetic acid affinity column as directed by the manufacturer (Qiagen).

Using primers, mlipo-F (5′- CATGCCATGGATGGGCGAAAAAGCGACTGAC-3′; the underlined sequence denotes an NcoI restriction site) and lipoR, we prepared the truncated *ilpA* gene, which lacked the 5′-end of DNA fragment (69 bp) containing the consensus sequence for lipidation. The resultant *ilpA′* DNA fragment of 741 bp was then used to clone into pET28b as described above. The truncated rIlpA protein was overexpressed in *E. coli* BL21 (DE3) carrying the resultant plasmid, pETmilpA, and purified in the same manner as the intact rIlpA protein.

Determination of the Recombinant IlpA Protein Lipidation—*In vivo* labeling of rIlpA protein with [^{3}H]palmitic acid was performed as described (33). Briefly, various *E. coli* strains, DH5α carrying pET28b, DH5α carrying pETilpA, BL21 (DE3) carrying pET28b, and BL21 (DE3) carrying pETilpA, were grown to the mid-log phase (A_{600} = 0.6–0.8), and the incubation was continued for 12 h in the presence of 50 μCi of [^{3}H]palmitic acid (Amersham Biosciences) without or with IPTG. After the labeling was stopped by adding 10% trichloroacetic acid (w/v), the labeled bacterial cells were resuspended in boiling buffer (2% (w/v) SDS, 50 mM Tris-Cl, pH 8.0), boiled for 10 min, and then broken by sonication. The bacterial extracts were then phase-partitioned into aqueous and detergent phases with the addition of 5% Triton X-114 at 37 °C. The pellet fraction was washed three times with radioimmunoprecipitation buffer (1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 50 mM Tris-Cl, pH 8.0) before being resuspended in sample buffer for SDS-PAGE. An equal microgram of each fraction was subjected to 10% (w/v) SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was treated with Amplify solution (Amersham Biosciences) and exposed to preflashed Hyperfilm-MP (Amersham Biosciences) for 2 weeks at −70 °C.

Western Blot Analysis—Purified rIlpA (100 μg) was mixed with 0.5 ml of complete Freund's adjuvant (Sigma), and injected intraperitoneally into a specific pathogen-free rat (CrjBgi: CD[S.D.]IGS, 7-week-old, female). Two additional immunizations were performed with the same amount of rIlpA protein mixed with incomplete Freund's adjuvant (Sigma) at 2 and 4 weeks after the primary immunization. A week after the third immunization, serum was obtained from the immunized rat and used for Western blot analysis.

Lysates of various *V. vulnificus*, wild type ATCC29307, YS101, and YS101 harboring pLAFRilpA, were prepared in a lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, and 20% (v/v) glycerol), separated in a 10% (w/v) SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore). The membranes were incubated with IlpA antisera (1:20,000) in a blocking solution (PBS with 5% (w/v) skim milk and 0.05% (v/v) Tween 20), washed three times with PBS containing 0.1% (v/v) Tween 20, and incubated with alkaline phosphatase-conjugated anti-rat immunoglobulin G. The immunoreactive bands were visualized by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate system (Promega).

Preparation of Human Peripheral Mononuclear Cells and Monocytes—Human peripheral mononuclear cells (PBMCs) were prepared by density gradient centrifugation using Ficoll (Amersham Biosciences) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2

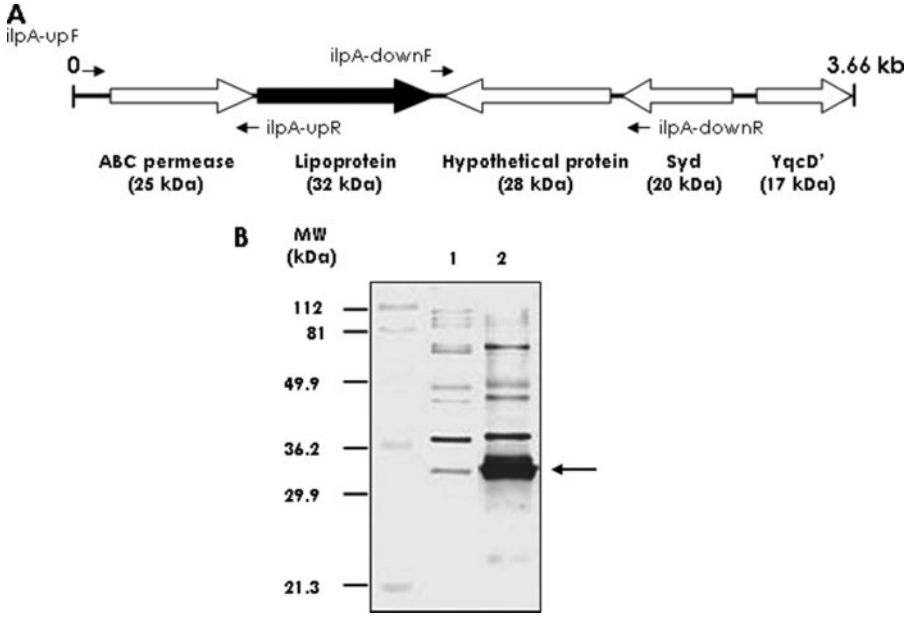

FIGURE 1. **Genetic organization of the insert DNA in plasmid pEX3, and identification of the gene encoding the protein that reacted with anti-OMPvv antibodies.** *A*, pEX3 is one of the immunoreactive clones, which was isolated by immunoscreening of the *V. vulnificus* expression library with the antibodies raised against OMPs of *V. vulnificus* (OMPvv). A 3.66-kb DNA fragment of pEX3 contains four complete ORFs that encode an ABC permease, a lipoprotein, a hypothetical protein, a SecY-interacting protein, and a partial ORF for a hypothetical protein. Sizes of the putative proteins are indicated in *parentheses*. Primers used for the construction and confirmation of a Δ*ilpA* mutant *V. vulnificus* are denoted with *arrows*. *B,* 20 μg of protein extracts from *E. coli* XLOLR and *E. coli* XLOLR carrying pEX3 was subjected to Western analysis using antibodies against OMPvv. An ~30-kDa protein appeared as an immunoreactive band and is indicated by an *arrow*. *Lane 1*, lysate prepared from XLOLR; and *lane 2*, lysate prepared from XLOLR carrying pEX3.

mM L-glutamine. Prepared PBMCs (1×10^5 cells) were cultured in 96-well plates. Monocytes (>95%) were isolated by countercurrent elutriation from peripheral blood as described previously (34). Certain numbers of monocytes, 1×10^5 or 5×10^5, were cultivated in 96-well or 24-well flat-bottom microtiter plates, respectively. Cells were cultured with or without *V. vulnificus* lysates, the rIlpA protein, concanavalin A, or LPS.

Measurement of Cytokines—PBMCs and monocytes in 96-well plates (1×10^5 cells per well) were coincubated with culture medium, bacterial lysates, or rIlpA protein at the indicated concentration for 18 h. For comparison, cells were also treated with commercially available *E. coli* LPS (Sigma). Cell-free supernatants were then collected by centrifugation, transferred to new tubes, and stored at −70 °C until analysis. The levels of each cytokine in the supernatants were determined using enzyme-linked immunosorbent assays (BD Biosciences). Concentration of each cytokine was determined based on the standard curve of the corresponding cytokine, which had been purified and was available commercially (IFN-γ from BD Biosciences; TNF-α and IL-6 from BIOSOURCE).

Blocking Experiments with Polymyxin B or with Anti-TLR2 Antibodies—Monocytes were preincubated for 1 h with medium containing 10 μg/ml human TLR2 monoclonal antibodies (BioLegend) or its isotype control IgG (BioLegend). To examine the role of residual LPS in the prepared rIlpA protein fraction on cytokine production by monocytes, polymyxin B (Sigma) was added to the rIlpA at a concentration of 20 μg/ml prior to being incubated with the prepared monocytes.

Transfection and Luciferase Assay—Using Lipofectamine (Invitrogen), human embryonic kidney 293 (HEK 293) cells (5×10^5) were transfected with 1.0 μg of pFLAG-TLR2 (35), 1.0 μg of $p(IL6\kappa B)_3$50hu.IL6P-luc+ (36), and 0.5 μg of pCH110 (GE Healthcare) in 24-well plates. As a control, the plasmid pFLAG-CMV (Invitrogen) was transfected instead of pFLAG-TLR2. pFLAG-TLR4 (35) was used to transfect HEK 293 cells along with $p(IL6\kappa B)_3$50hu.IL6P-luc+ and pCH110. After 24 h, the transfected cells were stimulated with either rIlpA or LPS. After an additional 20-h incubation, the cells were lysed and assayed for luciferase activity using a luciferase reporter assay system (Promega). Normalized reporter activity is expressed as the luciferase activities derived from $p(IL6\kappa B)_3$50hu.IL6P-luc+ divided by the activities of β-galactosidase derived from pCH110 of the same transfected cells.

RT-PCR of TLR mRNAs—Using TRIzol reagent (Invitrogen), total RNAs were isolated from human PBMCs (2×10^6) stimulated with either rIlpA or *E. coli* LPS (Sigma) at the concentration of 1.0 μg/ml. As a control, total RNA was also prepared from the same number of PBMCs. In addition, HEK293-hTLR2/CD14 (indicated as 293-hTLR2) and HEK293-hTLR4/MD2-CD14 (indicated as 293-hTLR4) cell lines (Table 1) were treated with either rIlpA or LPS and subjected to RNA preparation.

Two micrograms of each RNA were converted into cDNA using the oligo(dT) primer via the action of reverse transcriptase, which was then amplified for 22 cycles using specific sets of primers. Two primers for TLR2, TLR2-F (5′-GCCAAAGCTTTGATTGATTGG-3′) and TLR2-R (5′-TTGAAGTTCTCCAGCTCCTG-3′), were designed on the nucleotide sequences of human TLR2 (GenBank™ accession number NM003264), whereas TLR4-specific primers, TLR4-F (5′-TGCGGGTTCTACATCAAA-3′) and TLR4-R (5′-CCATCCGAAATTATAAGAAAAGTC-3′), were made based on the nucleotide sequence of human TLR4 (GenBank™ accession number U88880). Two oligonucleotides derived from the GAPDH sequence (5′-GGTCATCCCTGAGCTGAACG-3′ and 5′-TCCGTTGTCATACCAGGAAAT-3′) were used to amplify the control RNA. Each cycle of the PCR consisted of a denaturation step (94 °C, 30 s), an annealing step (52 °C, 30 s), and an elongation step (72 °C, 30 s). The PCR products were subjected to electrophoresis in a 1.8% agarose gel.

LD_{50} Determination—For determination of LD_{50}, specific pathogen-free, 7-week-old, female ICR mice were used without pretreatment with iron dextran. Cultures of bacterial strains grown overnight in LBS medium were freshly cultivated in the same medium up to an A_{600} of 0.7, harvested, washed once in PBS, and then resuspended in PBS. One hundred microliters of

serial dilutions of the bacterial suspension were then injected intraperitoneally into six mice per dilution group. The numbers of dead mice were determined 24 h after the injection, and the LD_{50} value was calculated using the equation described previously by Reed and Muench (37).

Nucleotide Sequence Accession Number—The nucleotide sequences of the *ilpA* gene isolated from *V. vulnificus* ATCC29307 were deposited in the GenBank™ data base under the accession number of DQ177330.

Statistical Analysis—Data are presented as means ± S.D. derived from at least three independent experiments. Student's *t* test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.) was used to evaluate the statistical significance of the results.

RESULTS

Isolation of the Immunoreactive Clone from Immunoscreening V. vulnificus OMPs—Approximately 30,000 plaques from the λZAPII-based expression library of *V. vulnificus* were screened to isolate the clones expressing the proteins reacting with the antibodies against *V. vulnificus* OMPs. Twelve candidate plaques showing reproducible immune response with anti-OMP serum were isolated, and the insert DNAs in the isolated phages were excised to pBK-CMV phagemids. Among the 12 isolated clones, 3 of them, pEX3, pEX19, and pEX21, had an identical 3.66-kb DNA fragment (Fig. 1*A*). The DNA insert of pEX3 was found to contain four complete ORFs coding for an ABC permease, a putative lipoprotein, a hypothetical protein, a SecY-interacting protein (Syd), and a partial ORF encoding another hypothetical protein.

To examine which gene product was responsible for the immune reaction with anti-OMP serum, the lysate of *E. coli* XLOLR containing pEX3 was subjected to a Western blot analysis using anti-OMP serum (Fig. 1*B*). As a control, lysates of *E. coli* XLOLR without any plasmid were reacted with the same serum. A strong immunoreactive band of ~30 kDa was detected only in the lysate of XLOLR with pEX3. The molecular mass of this immunoreactive protein, ~30 kDa, is a similar size to the tentative protein encoded by the second ORF in pEX3, which codes for a putative lipoprotein. Thus, this putative lipoprotein may be an antigenic molecule of *V. vulnificus* found in the immunoscreening assays.

Blast search of this putative lipoprotein reveals several homologous proteins found in other pathogenic Gram-negative bacteria (Fig. 2). The deduced amino acid sequences of this *V. vulnificus* lipoprotein (GenBank™ accession number DQ_177330) showed 84, 68, 53, and 59% identities to those of *Vibrio cholerae* (GenBank™ accession number NP_230552), *Salmonella typhimurium* (GenBank™ accession number NP_459249), *E. coli* (GenBank™ accession number YP_859264), and *Haemophilus influenzae* (GenBank™ accession number P_317282), respectively. None of these proteins has yet been reported with respect to their functional role. In all five proteins, however, there is a conserved signal sequence and a cysteine residue, which have been known to be required for lipidation (38). Therefore, we putatively designated this protein as IlpA, which stands for immunogenic lipoprotein A.

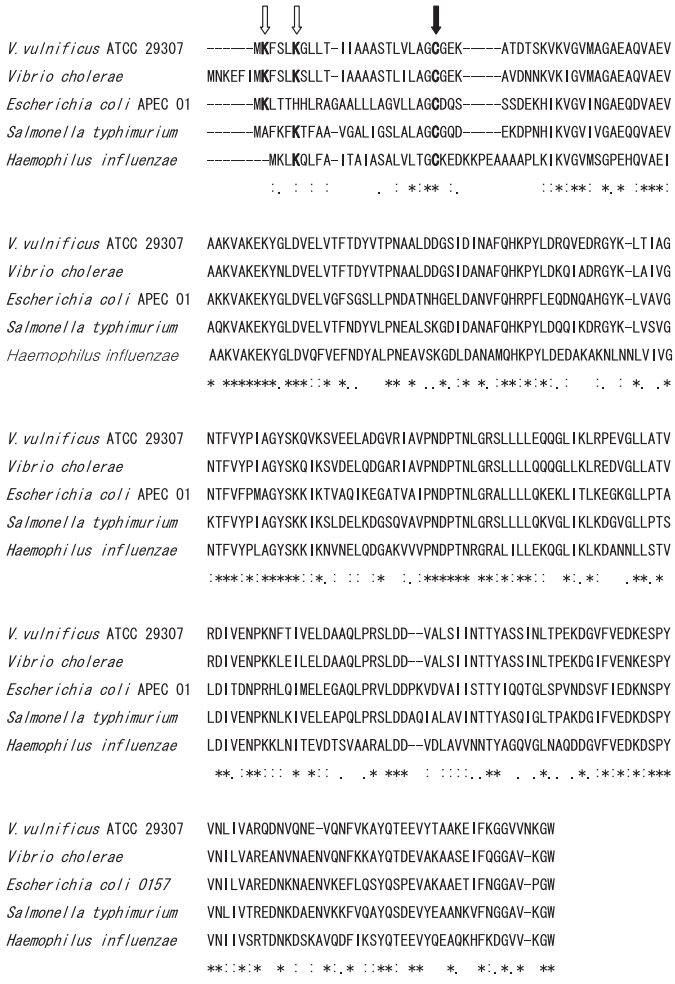

FIGURE 2. **Alignment of the deduced lipoprotein amino acid sequences of *V. vulnificus*, *V. cholerae*, *E. coli*, *S. typhimurium*, and *H. influenzae*.** The signal sequences for protein secretion and the positively charged lysine within the N-terminal hydrophobic region, are indicated by *open arrows*, and the cysteine residue for lipidation is denoted by a *closed arrow*. An *asterisk* indicates a fully conserved residue. The *two-dotted symbol* indicates a strongly similar residue, and the *one-dotted symbol* indicates a weakly similar residue.

Lipidation of the Recombinant IlpA Protein—The deduced amino acid sequences of this putative protein contain a consensus lipobox sequence for lipid modification at its N terminus, which is Leu-Ala-Gly(Ala)-Cys (38). Using an expression system of the rIlpA in *E. coli*, we investigated whether IlpA protein is modified by lipidation as suggested from its amino acid sequences (Fig. 3). The rIlpA protein was expressed in *E. coli* BL21 (DE3) as a C terminus histidine-tagged form using the pET28b-based expression plasmid, pETilpA, in which the expression of the recombinant protein is induced by IPTG. *E. coli* DH5α was also used as a control strain, in which expression of the rIlpA was not induced by IPTG.

Four different *E. coli* strains, DH5α with pET28b, BL21 (DE3) with pET28b, DH5α with pETilpA, and BL21 (DE3) with pETilpA, were grown in the presence of [^{3}H]palmitic acid and examined for the presence of radiolabeled protein(s). Specifically, two of the four strains, DH5α carrying pETilpA and BL21 (DE3) carrying pETilpA, were cultured in the absence or presence of 1 mM IPTG and then compared side by side to examine

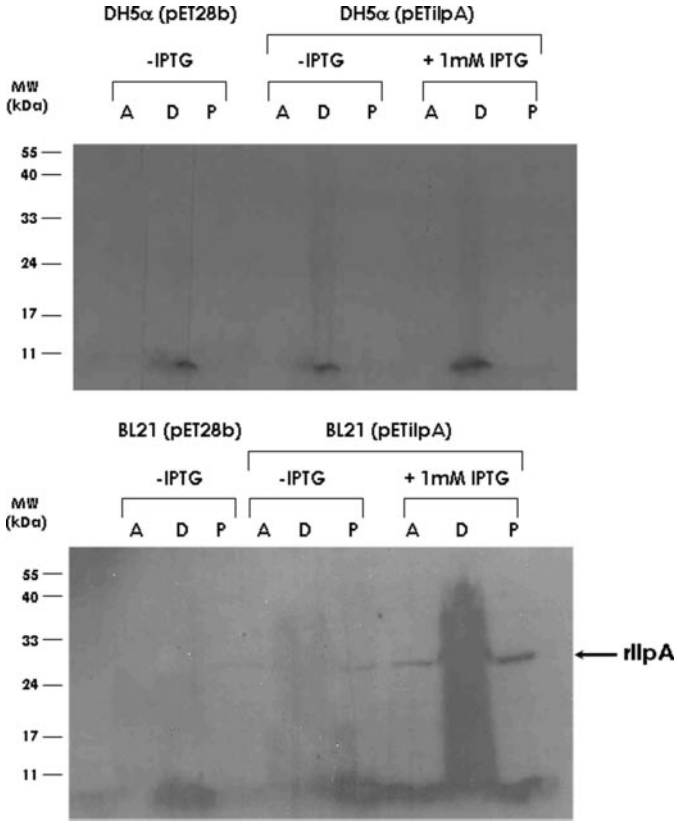

FIGURE 3. **Autoradiograph of the rIlpA labeled with [^{3}H]palmitic acid.** Various *E. coli* strains (DH5α carrying pET28b, DH5α carrying pETilpA, BL21 (DE3) carrying pET28b, and BL21 (DE3) carrying pETilpA) at mid-log phase were labeled with [^{3}H]palmitic acid. Bacterial lysates were then phase-partitioned with 5% (v/v) Triton X-114 at 37 °C. For two strains, DH5α carrying pETilpA and BL21 (DE3) harboring pETilpA, the expression of the recombinant protein was induced by adding 1 mM IPTG. An equal amount of each fraction was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was treated with Amplify solution (Amersham Biosciences) and exposed to x-ray film for 2 weeks. *A,* aqueous phase; *D,* detergent phase; *P,* pellet. The ^{3}H-labeled rIlpA is indicated by an *arrow*.

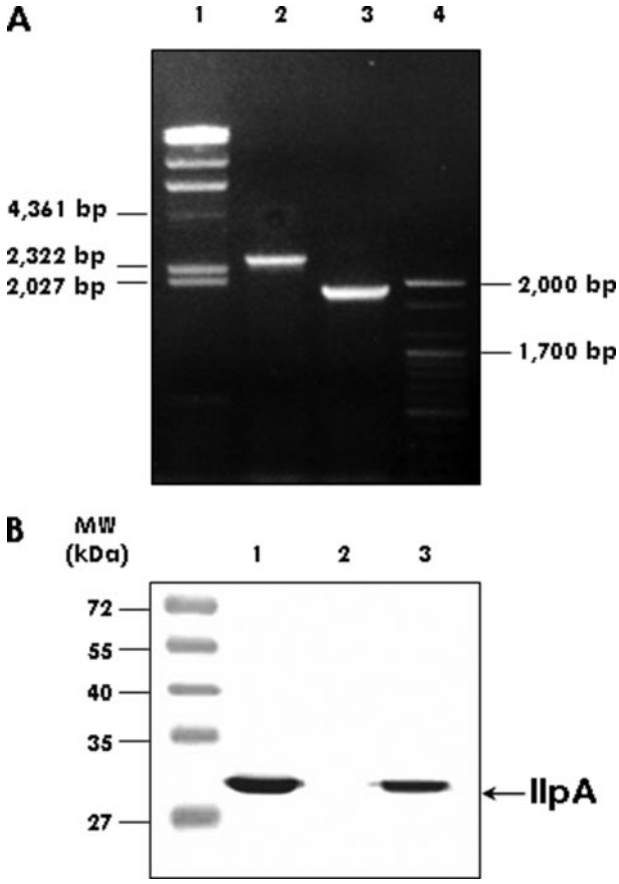

FIGURE 4. **Confirmation of Δ*ilpA* mutant strain (YS101) and its complemented strain of *V. vulnificus*.** *A,* deletion of the *ilpA* locus in the strain YS101 was confirmed by PCR using a set of primers, ilpA-upF and ilpA-downR (Fig. 1). *Lane 1*, DNA size marker (HindIII-digested λ phage DNA); *lane 2*, PCR product of wild type ATCC29307; *lane 3*, PCR product of YS101; and *lane 4*, DNA size marker (100 bp ladder). *B,* Western blot of various *V. vulnificus* strains using polyclonal antibodies specific to rIlpA. Each lane contains 20 μg of bacterial lysate prepared from wild type ATCC29307 (*lane 1*), the Δ*ilpA* mutant (*lane 2*), or the Δ*ilpA* mutant complemented with the intact *ilpA* gene in pLAFR5 (*lane 3*). An immunoreactive band of ~30 kDa is indicated by an *arrow*.

whether the IPTG-induced protein might be labeled with [^{3}H]palmitic acid. Because proteins modified by lipidation have detergent-soluble properties, lysates of each culture were subdivided into aqueous, detergent, and pellet fractions by their solubility in Triton X-114.

A protein labeled with [^{3}H]palmitic acid, the size of which is ~30 kDa, was detected only in the lysates prepared from BL21 (DE3) with pETilpA, when grown in the presence of IPTG. This result indicates that the rIlpA protein is lipidated as suggested from its amino acid sequences. The lipid-containing property of the rIlpA was also supported by observing the strongest radioactivity in the detergent phase of IPTG-induced BL21 (DE3) bacteria with pETilpA.

Construction of ilpA Knock-out Mutant and Complementation of the Mutant—We constructed a mutant *V. vulnificus*, in which its *ilpA* gene was deleted from the wild type *V. vulnificus* ATCC29307 to examine the functional role of this protein in *V. vulnificus*. Two sets of primers were used to construct a knock-out mutant, *i.e.* a set of two primers specific to the upstream region (ilpA-upF and ilpA-upR) and a second set of primers specific to the downstream region (ilpA-downF and ilpA-downR) of the *ilpA* gene (Fig. 1*A*). The resultant mutant, YS101, lacked a complete ORF of the *ilpA* gene. Deletion of the *ilpA* gene in a chromosome of the mutant *V. vulnificus* was confirmed by PCR using primers ilpA-upF and ilpA-downR (Fig. 4*A*). The resultant PCR product of the *ilpA* mutant *V. vulnificus* was 1,855 bp long, whereas wild type *V. vulnificus* with the intact *ilpA* gene produced a larger PCR product of 2,665 bp.

The cellular level of the IlpA protein was also examined in wild type and Δ*ilpA* mutant *V. vulnificus* by Western blot analysis using polyclonal antibodies against rIlpA (Fig. 4*B*). A strong immunoreactive band of ~30 kDa was detected only in the wild type lysate. An intact *ilpA* gene of *V. vulnificus* was cloned into the broad host range plasmid pLAFR5 to produce pLAFRilpA. The Δ*ilpA* mutant *V. vulnificus* carrying pLAFRilpA clearly demonstrated a formation of the immunoreactive protein (Fig. 4*B*).

Cytokine Production of Human PBMCs Induced by Cell Extracts of Various V. vulnificus Strains—Repeated isolation of *ilpA*-containing clones from immunoscreening of *V. vulnificus* expression library with anti-OMP serum led us to examine the possibility that IlpA of *V. vulnificus* acts as an immunostimulant to induce cytokine production in human cells. Thus, we prepared a *V. vulnificus* lysate devoid of the IlpA protein using

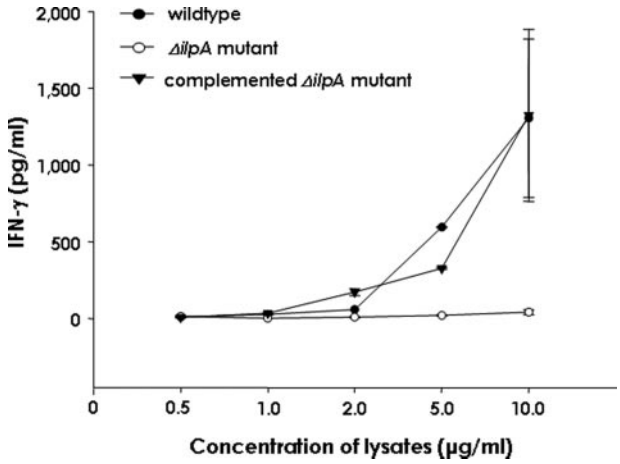

FIGURE 5. **Cytokine production of human PBMCs induced by *V. vulnificus* cell lysates.** PBMCs were isolated by centrifugation of heparinized human blood over a Ficoll-Paque density gradient at 1,600 rpm for 30 min. PBMCs (1×10^5) were then incubated with various concentrations of bacterial lysates up to 10.0 μg/ml for 18 h. The concentrations of IFN-γ stimulated by lysates of wild type, Δ*ilpA* mutant, and complemented Δ*ilpA* mutant *V. vulnificus* were determined using human IFN-γ ELISA kit (BD Biosciences) and indicated as *closed circles, open circles*, and *closed inverted triangles*, respectively. *Error bars* represent the standard deviations from three independent experiments. IFN-γ production induced by each lysate of *V. vulnificus* was measured in triplicate for each experiment.

the Δ*ilpA* mutant YS101 and compared its activity to induce cytokine production with that of wild type *V. vulnificus*. When human PBMCs were treated with various concentrations of wild type lysate (ranged from 0.5–10.0 μg/ml), the secretion of IFN-γ by PBMCs was increased up to 1,310 pg/ml in a dose-dependent manner (Fig. 5). In contrast, treatment of PBMCs with the lysate of the Δ*ilpA* mutant *V. vulnificus* produced a much lower level of IFN-γ (41 pg/ml), which was a comparable value to those of the control cells, such as PBMCs treated with medium only or bovine serum albumin (13 or 32 pg/ml, respectively).

When lysate of the Δ*ilpA* mutant *V. vulnificus* carrying pLAFRilpA was used to challenge PBMCs, IFN-γ production was restored to the levels induced by the equivalent concentrations of wild type *V. vulnificus* lysate. This result suggests that *V. vulnificus* IlpA protein stimulates an immune response in human cells.

Cytokine Production of Human PBMCs and Monocytes Induced by rIlpA Protein—Based on the drastic difference in immunogenically stimulating activity between lysates of wild type and Δ*ilpA* mutant *V. vulnificus*, we asked whether the IlpA protein itself was able to induce production of IFN-γ in PBMCs (Fig. 6*A*). Indeed, when rIlpA protein was added at a concentration of 1.0 μg/ml to PBMCs, IFN-γ was increased to 1,080 pg/ml. This was equivalent to the IFN-γ level in a positive control (1,320 pg/ml), in which PBMCs were treated with 1.0 μg/ml concanavalin A, a well known molecule to induce production of IFN-γ (39).

In subsequent experiments, we examined the ability of rIlpA to induce the production of cytokines in monocytes. Monocytes prepared from human PBMCs were challenged with various concentrations of rIlpA (0.01–1.0 μg/ml) and assayed for production of TNF-α (Fig. 6*B*) and IL-6 (Fig. 6*C*). As a positive control, *E. coli* LPS was used to stimulate cytokine production by monocytes. When these rIlpA-treated monocytes were

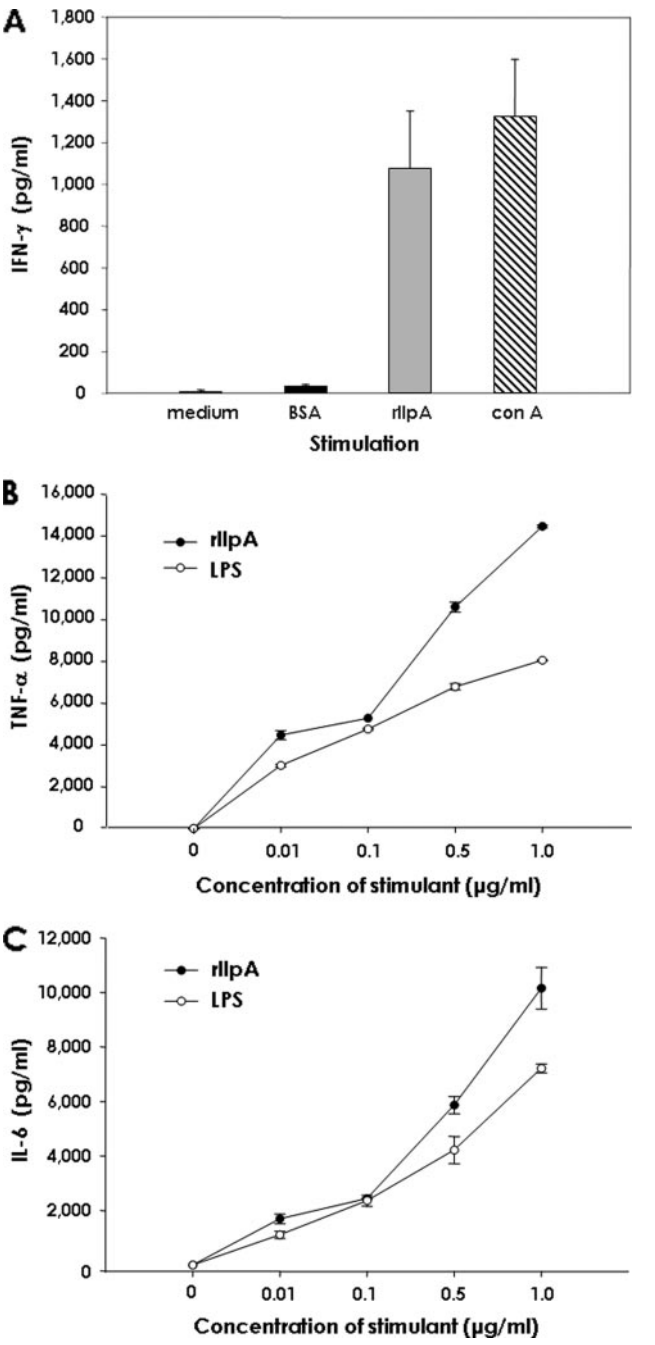

FIGURE 6. **Cytokine production induced by rIlpA of *V. vulnificus*.** *A,* prepared PBMCs (1×10^5) were incubated with 1.0 μg/ml rIlpA for 18 h, and the supernatant of each culture was used to determine the level of IFN-γ production using a human IFN-γ ELISA kit (BD Biosciences). PBMCs exposed to concanavalin A (*con A*) (1.0 μg/ml) were included as a positive control for IFN-γ production. In addition, PBMCs were also treated with medium or bovine serum albumin and assayed for cytokine production as negative controls. *B* and *C,* confluent monocytes (1×10^{5}/ml), which were prepared as described under "Experimental Procedures," were exposed to various concentrations of rIlpA protein ranging from 0.01 to 1.0 μg/ml. Monocytes treated with *E. coli* LPS were used as a positive control for cytokine production. Cytokine concentrations were measured using a human TNF-α ELISA kit (BIOSOURCE) and a human IL-6 ELISA kit (BIOSOURCE). *Error bars* represent the standard deviations from three independent experiments. Measurement of each cytokine induced by rIlpA was performed in triplicate for each experiment.

assayed for cytokine production at 18 h post-stimulation, increasing amounts of both cytokines were detected in a dose-dependent manner of rIlpA (up to 14,500 pg/ml for TNF-α and

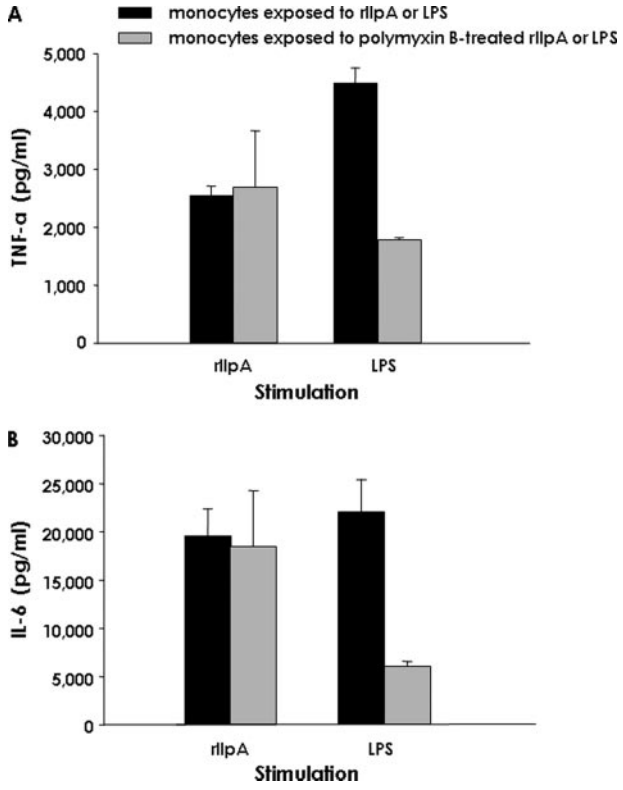

FIGURE 7. **Effect of polymyxin B on cytokine production by rIlpA-treated monocytes.** Polymyxin B was added to rIlpA at a concentration of 20 μg/ml to neutralize residual LPS, which might be coeluted during the purification process for rIlpA. Monocytes (5×10^5) were seeded in 24-well culture plates and exposed to rIlpA protein or to polymyxin B-treated rIlpA at a concentration of 1.0 μg/ml. LPS of *E. coli* was also treated simultaneously with polymyxin B to provide a control for the neutralizing effect of polymyxin B against LPS. Following an 18-h exposure to the rIlpA protein or LPS, the culture supernatants were collected and then assayed for TNF-α (*A*) or IL-6 (*B*) by ELISA as described under "Experimental Procedures." *Error bars* represent the standard deviations from three independent experiments. Induction of each cytokine by IlpA, polymyxin B-treated rIlpA, LPS, or polymyxin B-treated LPS was performed in triplicate for each experiment.

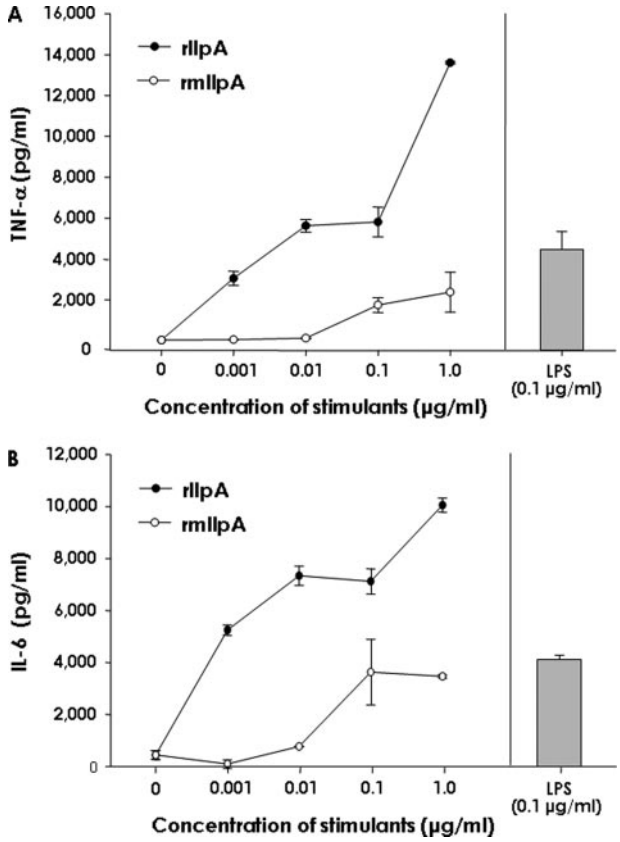

FIGURE 8. **Role of the lipid moiety of rIlpA in cytokine production by monocytes.** Confluent monocytic cells (1×10^5/ml) were exposed for 18 h to various concentrations of the wild type rIlpA protein or nonlipidated mutant rIlpA protein (rmIlpA) ranging from 0.01 to 1.0 μg/ml. LPS of *E. coli* was used as a positive control for induction of cytokine production in monocytes. Culture supernatants were then collected and assayed for TNF-α (*A*) and IL-6 (*B*) by ELISA as described under "Experimental Procedures." *Error bars* represent the standard deviations from three independent experiments. Production of each cytokine by wild type rIlpA or rmIlpA at various concentrations was measured in triplicate for each experiment.

10,200 pg/ml for IL-6). In response to the increased amount of LPS, monocytes displayed increased formation of these two cytokines, up to 8,050 pg/ml for TNF-α and 7,220 pg/ml for IL-6. These results indicate that rIlpA of *V. vulnificus* is as potent as *E. coli* LPS in inducing cytokine production by monocytes.

Treatment of rIlpA Protein with Polymyxin B—LPS of Gram-negative bacteria is known as a potent immunostimulant (40), and the rIlpA protein used in this experiment was prepared from the Gram-negative bacterial cell, *E. coli* BL21 (DE3). Thus we examined the possibility that cytokine production by rIlpA-stimulated monocytes was caused from the residual contamination of LPS in the purified fraction of rIlpA. To exclude this possibility, polymyxin B, an amphipathic LPS-binding molecule, was used to antagonize the function of LPS. The polymyxin B-treated rIlpA was used to stimulate monocytes, upon which the production of TNF-α (Fig. 7*A*) and IL-6 (Fig. 7*B*) was determined. As a positive control to confirm the proper functioning of polymyxin B, LPS was also pretreated with polymyxin B before it was used for monocyte activation. As expected, the ability of polymyxin B-treated LPS to stimulate cytokine production in monocytes was dramatically attenuated compared with the ability of untreated LPS-stimulated monocytes. In contrast, preincubation of rIlpA with polymyxin B did not affect the ability of rIlpA to induce cytokine production in monocytes. This result clearly demonstrates that the rIlpA protein used in this experiment was free of LPS contamination, and the cytokine production by rIlpA-exposed monocytes is derived from immunogenic activity of IlpA of *V. vulnificus*.

Role of Lipid Moiety of IlpA in Cytokine Production in Monocytes—The amino acid sequence of IlpA has a putative sequence for lipid modification, and lipidation of rIlpA was confirmed by *in vivo* labeling assay (Fig. 3). In the case of the *Borrelia burgdorferi* surface lipoprotein, its lipid moiety has been reported to be crucial for immunogenic function (41). To examine the role of the IlpA lipid moiety in cytokine production by monocytes, we synthesized a truncated rIlpA protein, in which 23 N-terminal residues were deleted. Because the missing region of the mutant rIlpA protein contains important amino acid residues required for lipidation, this protein was not expected to be modified by lipidation, as shown previously in other lipoproteins (41). Monocytes were stimulated with the mutant rIlpA at a various concentrations from 0.001 to 1.0 μg/ml, and then assayed for production of TNF-α (Fig. 8*A*) and

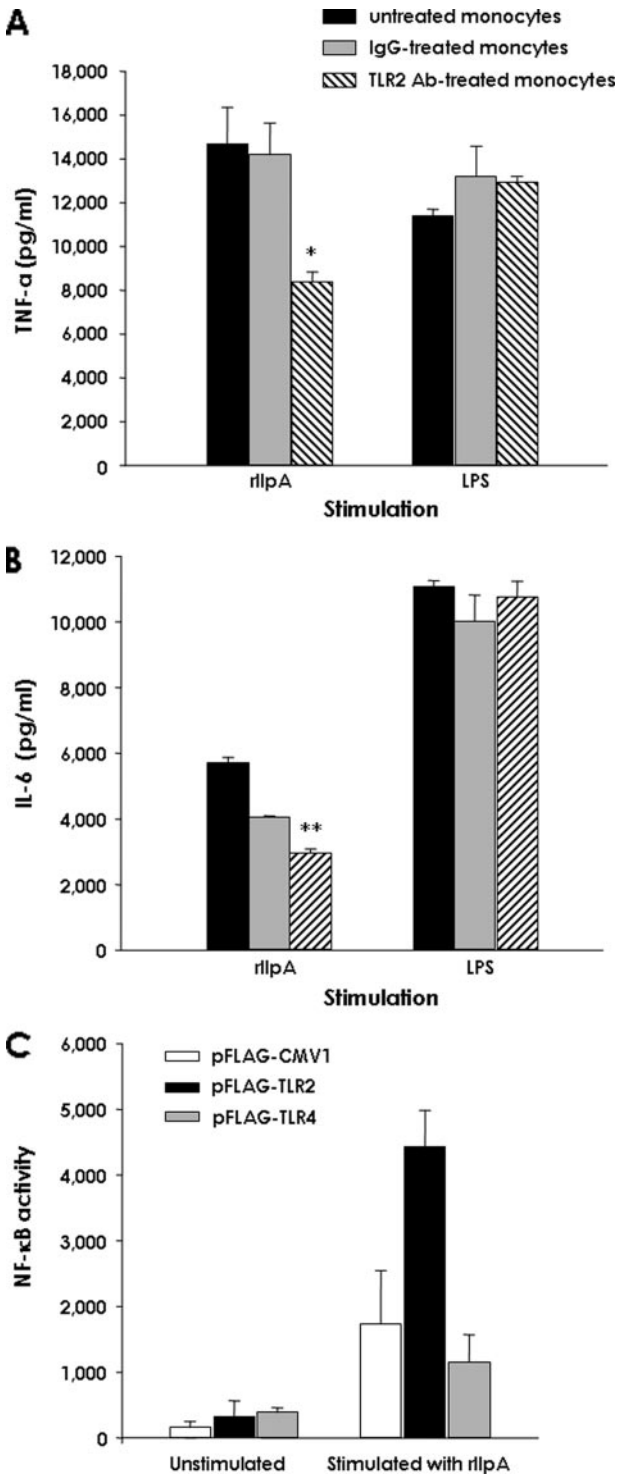

FIGURE 9. **Role of TLR2 in cytokine production by rIlpA-stimulated monocytes.** *A* and *B*, human monocytes were preincubated for 1 h with either anti-TLR2 antibodies or with the relevant isotype IgG control. These cells were then stimulated with rIlpA (1.0 μg/ml) for 18 h, and then culture supernatants were assayed for TNF-α (*A*) and IL-6 (*B*) by ELISA as described under "Experimental Procedures." A control set of monocytes pretreated with either the TLR2 antibodies or with the control IgG was stimulated with LPS instead of rIlpA and then assayed for cytokine production. *C*, HEK 293 cells (5×10^5), which have been transfected with pFLAG-TLR2, p(IL6κB)$_3$50hu.IL6P-luc+, and pCH110, were stimulated with rIlpA (1.0 μg/ml), incubated for an additional 20 h, and lysed to determine both luciferase and β-galactosidase activities. The NF-κB activities were shown by dividing the luciferase activities (relative light units) by the β-galactosidase activities of the identical sample. As controls, plasmid pFLAG-CMV or pFLAG-TLR4 was included in the transfection experiments. *Error bars* represent the standard deviations from three independent experiments. Production of IL-6 or TNF-α was measured in triplicate for each sample. *Asterisks* indicate cytokine levels that were significantly different from that of untreated monocytes by the Student's *t* test. Data with a *p* value of <0.01 are indicated with **, and data with a *p* value between 0.01 and 0.05 are represented with *.

IL-6 (Fig. 8*B*). As a control, another set of monocytes was treated with intact IlpA at the same concentrations as the mutant IlpA, and then assayed for cytokine production. When monocytes were treated with the mutant rIlpA at low concentrations (less than 0.1 μg/ml), only basal levels of cytokines were detected. Formation of significant amounts of cytokines was observed in monocytes stimulated with relatively high concentrations of the mutant rIlpA (0.1 and 1.0 μg/ml). On the other hand, considerable amounts of both TNF-α and IL-6 were formed in monocytes challenged with even the lowest concentration (0.001 μg/ml) of the intact IlpA, and cytokine production increased in proportion with IlpA concentration as shown in Fig. 6. Monocytes exposed to 0.1 μg/ml LPS were also examined for their induction of TNF-α and IL-6 production to monitor the proper experimental procedures, such as a stimulus treatment and cytokine assays. This result indicates that the nonlipidated mutant rIlpA was significantly impaired in its ability to induce cytokine production in monocytes compared with the ability of intact lipidated IlpA.

Role of TLR2 in Cytokine Production by IlpA-stimulated Monocytes—In this experiment, we investigated how the IlpA protein of *V. vulnificus* was recognized by human monocytes to result in cytokine production. In particular, we examined whether IlpA-induced cytokine production is mediated by a TLR family member, *e.g.* well known receptors for pathogen-associated molecular patterns, which relay information with respect to microbial components resulting in innate immunity. In *Mycobacterium tuberculosis*, antigenic lipoproteins were reported to be recognized by TLR2 (42). In addition, TLR2, which has been reported to function in inflammatory events associated with Lyme arthritis, recognizes lipoproteins of *B. burgdorferi* (43). Thus, we examined whether the IlpA of *V. vulnificus* also activates human monocytes via its interaction with TLR2.

To examine the possibility that TLR2 is involved in IlpA-induced cytokine production of monocytes, monocytes were pretreated with antibodies specific to TLR2 prior to being stimulated with rIlpA. As a control, another set of monocytes was pretreated with isotype control IgG instead of TLR2 antibodies, and then stimulated with rIlpA by the identical manner. These rIlpA-challenged monocytes were then assayed for production of TNF-α (Fig. 9*A*) and IL-6 (Fig. 9*B*). As shown in the previous figures, both TNF-α and IL-6 were produced in monocytes challenged with rIlpA. Pretreatment of monocytes with the IgG control did not affect the formation of these two cytokines by rIlpA. In contrast, cells pretreated with TLR2 antibodies showed significant decrease in both TNF-α and IL-6 production compared with isotype IgG-treated monocytes ($p < 0.02$, Student's *t* test).

LPS is known to be recognized by another TLR, TLR4 (26, 44). Thus, another set of monocytes was challenged with LPS

instead of rIlpA to determine whether the neutralizing activity of anti-TLR2 antibodies occurred only in rIlpA-treated cells but not in LPS-treated cells. Both the isotype IgG and anti-TLR2 antibodies were ineffective in inhibiting cytokine production in LPS-stimulated monocytes. This result suggests that the IlpA protein of *V. vulnificus* may act as an immunostimulant to monocytes by being recognized via TLR2.

To investigate the function of TLR2 in IlpA-induced cytokine production in human monocytes, we also reconstituted an *in vitro* system using HEK 293 cells, in which either TLR2 or TLR4 was expressed along with their common adaptor protein MyD88 (45). As a control, another set of HEK 293 cells was transfected with pFLAG-CMV1, the empty vector for pFLAG-TLR2 (a TLR2-expressing plasmid; see Ref. 35) or pFLAG-TLR4 (a TLR4-expressing plasmid; see Ref. 35). To monitor cytokine production using a reporter gene, this system includes a luciferase reporter for NF-κB activities, $p(IL6\kappa B)_3$50hu.IL6P-luc+ (36). For each transfection, the *lacZ*$^+$-plasmid pCH110 was included to normalize luciferase activities by dividing with the β-galactosidase activities of the same transfectant.

The ability of rIlpA to induce cytokine production was monitored by determining luciferase activities of the TLR2-expressing HEK 293 cells, the TLR4-expressing HEK 293 cells, and the control HEK 293 cells (Fig. 9*C*). In the absence of rIlpA, all of the HEK 293 cells showed basal levels of the luciferase activities. Upon stimulation with rIlpA, all three HEK 293 cells showed increased luciferase activities. Interestingly, the HEK 293 cells transfected with pFLAG-TLR2 demonstrated the most dramatic increase in their NF-κB activities. On the contrary, the increase in luciferase activity was significantly less in the TLR4-expressing cells and the control HEK 293 cells than the TLR2-expressing HEK 293 cells.

Induction of TLR2 Transcription in Human PBMC and 293-hTLR Cell Lines by IlpA Treatment—To determine whether TLRs are expressed in the response to IlpA protein of *V. vulnificus*, we examined the levels of TLR2 and TLR4 mRNAs in the IlpA-treated cells by RT-PCR. When human PBMCs were challenged with rIlpA (1.0 μg/ml) for 1 h, the level of TLR2 mRNA was significantly increased (Fig. 10*A*). On the other hand, LPS-treated PBMC demonstrated increased levels of TLR4 mRNA at 5 h of post-stimulation (Fig. 10*A*). IlpA did not affect the level of TLR4 mRNA, and LPS did not influence the TLR2 mRNA in PBMC.

Two 293-hTLRs cell lines expressing either TLR2 or TLR4 were also examined for their response to IlpA by RT-PCR (Fig. 10*B*). Expression of TLR2 was increased in 293-hTLR2 cells at 1 h of exposure to rIlpA, whereas it was not affected by LPS stimulation at all. The amount of TLR4 mRNA was dramatically increased at 5 h after LPS stimulation in 293-hTLR4 cells. On the other hand, the level of TLR4 mRNA was only slightly increased by rIlpA treatment at 5 h in the 293-hTLR4 cells. The levels of GAPDH mRNA were examined as the controls for RNA amount.

Role of IlpA in Lethality of V. vulnificus to Mice—We examined the role of the IlpA protein in pathogenesis of *V. vulnificus* by using a mouse infection model. Upon intraperitoneal injection of various numbers of bacterial cells into mice, the numbers of dead mice were determined at 24 h after the injection

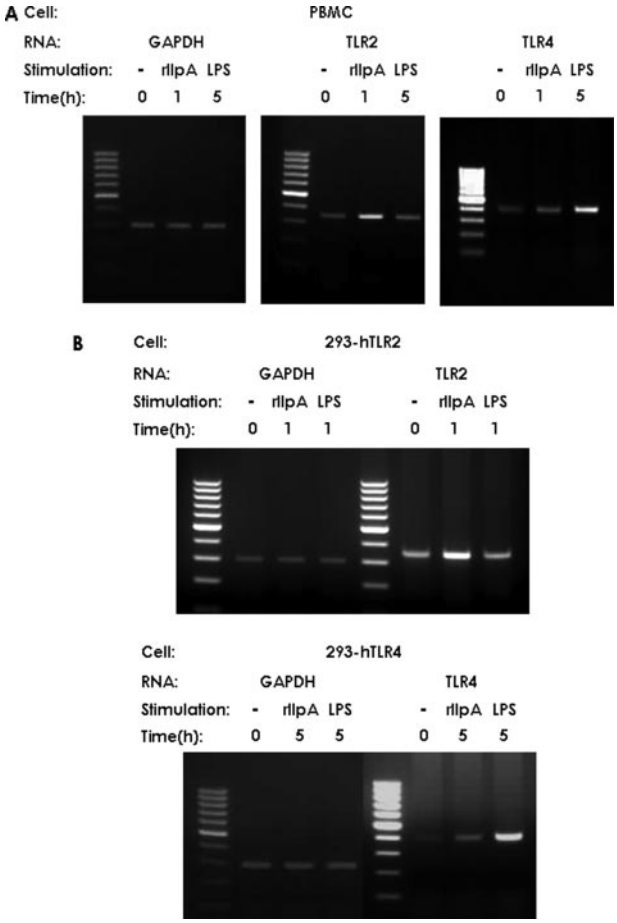

FIGURE 10. **Induction of Toll-like receptor mRNAs in human PBMCs, 293-hTLR2, and 293-hTLR4 cells.** *A,* total RNA was prepared from PBMCs (2×10^6) incubated with 1.0 μg/ml rIlpA for 1 h or with 1.0 μg/ml LPS for 5 h, and analyzed for their transcript levels of GAPDH, TLR2, and TLR4 using RT-PCR as described under "Experimental Procedures." *B,* levels of TLR2 mRNA and TLR4 mRNA were monitored by RT-PCR in 293-hTLR2 and 293-hTLR4 cells, which were pretreated with rIlpA or *E. coli* LPS. cDNA from a single RT reaction (derived from either 293-hTLR2 or 293-hTLR4) was used as a template for individual PCR with TLR2-specific primers or with TLR4 specific primers, respectively. Specific amplicons were visualized by 1.8% agarose gel electrophoresis and staining with ethidium bromide. GAPDH mRNA encoding glyceraldehyde-3-phosphate dehydrogenase was served as a loading control for RNA amount.

TABLE 2
Lethality of *V. vulnificus* strains to mice

Strain	Genotype	No. of dead mice/no. of total mice for no. of injected bacteria				LD_{50}
		3.0×10^3	3.0×10^4	3.0×10^5	3.0×10^6	
						CFU
ATCC29307	Wildtype	0:6	1:6	5:6	6:6	9.5×10^4
YS101	Δ*ilpA*	0:6	0:6	1:6	6:6	7.5×10^5

[a] CFU indicates colony-forming units.

(Table 2). One of two experiments was presented as a representative experiment. Mice infected with the wild type showed an LD_{50} of 9.5×10^4 cells, whereas mice injected with the Δ*ilpA* mutant showed an 8-fold higher LD_{50} (7.5×10^5 cells).

DISCUSSION

Human innate immune system recognizes microbial pathogens via action of its highly conserved pattern recognition receptors for microbial molecules, including LPS, peptidogly-

cans, lipoteichoic acids, and lipoprotein. Lipoproteins are produced by both Gram-negative and Gram-positive bacteria, and some lipoproteins are known as the abundant proteins in certain Gram-negative bacteria (19).

Lipoproteins of several microorganisms are known to activate a variety of host cells to produce cytokines. In *Mycoplasma* lacking a cell wall, surface lipoproteins such as VlpA and VlpC have been reported to induce cytokine production in macrophages (46). Several lipoproteins, LpqH, LpqG, and LprA, have been documented in *M. tuberculosis* to trigger innate inflammation (43, 47, 48). In addition, the signaling pathway involved in lipoprotein-induced activation of immune cells was found to be distinct from that involved by the LPS-induced process (49).

In this study, a lipoprotein of *V. vulnificus* was identified as a surface protein showing a potent antigenic property (Fig. 1). Therefore, we examined if this protein was able to stimulate the human cells to produce cytokine. Because the intact *V. vulnificus* exhibits too strong a cytotoxicity toward various types of human cells,[3] it is not applicable to measure cytokine production by directly treating PBMCs with intact *V. vulnificus*. Thus, bacterial lysates or recombinant proteins were used in this study. Indeed, wild type *V. vulnificus* lysate was effective to trigger cytokine production by human PBMCs, but *V. vulnificus* lysate lacking the isolated lipoprotein was significantly defective in cytokine induction (Fig. 5). Based on an observation that this lipoprotein of *V. vulnificus* functions as a major immunostimulant, we designated this protein as IlpA, which stands for immunogenic lipoprotein A. This is the first report on a lipoprotein of *Vibrio* spp. with immunostimulating activity. The lipoprotein from other Gram-negative bacteria, such as *N. gonorrhoeae* and *Legionella pneumophila* (50, 51), have the ability to stimulate the production of proinflammatory mediators in mammalian cells.

TLRs, a group of well known receptors of the innate immune response, contain a cytoplasmic domain, which is homologous to the signaling domain of the IL-1 receptor. Each TLR senses the presence of the specific components of pathogens and transfers the information to a signaling pathway leading to the activation of NF-κB. Activation of NF-κB results in the transcription of genes coding for various cytokines (24). In the case of lipoproteins, TLR2 has been found as a main cellular receptor in several pathogenic bacteria. For example, a 19-kDa lipoprotein of *Mycobacterium* has been shown to trigger the signaling pathway of the host cells primarily through TLR-2, resulting in a production of IL-12/NO by monocytes/macrophages and an apoptosis of monocytes (42, 52). In the case of *V. vulnificus* IlpA, it activates monocytes to produce TNF-α and IL-6 in a similar manner as LPS (Fig. 6). The receptor for Gram-negative bacterial LPS has been shown to be TLR4 (26). Therefore, the receptor for IlpA on monocytes was examined using two independent assays that clearly demonstrated the involvement of TLR2 in IlpA-mediated cytokine induction in monocytes. Cytokine production in rIlpA-stimulated monocytes is blocked by pretreatment with antibodies specific to human TLR2 (Fig. 9, *A* and *B*). In addition, *V. vulnificus* rIlpA induced activation of NF-κB only in the HEK 293 cell line expressing TLR2 (Fig. 9*C*), whereas it did not induce NF-κB activation in HEK 293 cells expressing TLR4.

Lipoproteins are present mainly in two forms, diacylated or triacylated (53). At present, biochemical properties of lipoproteins were extensively studied in *Mycoplasma* spp. (54). *Mycoplasma gallisepticum* and *Mycoplasma mycoides* have both diacylated and triacylated forms of lipoproteins (53). In TLR1-deficient mice, the macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP-2) containing two acyl chains failed to induce cytokine production, whereas *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl] (Pam3)/Cys-Ser-Lys-4 (CSK4) (Pam_3CSK_4) containing three acyl chains induced cytokine formation (55), suggesting that only triacylated lipoproteins are recognized by the combined action of TLR1 and TLR2. *In vivo* research using TLR6-knock-out mice indicates that diacylated lipoproteins are recognized by TLR6 in conjunction with TLR2 (56). Our data indicated that TLR2 is the main receptor responsible for *V. vulnificus* IlpA-mediated immune response. However, a role for other TLRs in this process is not yet known, and thus future investigation will be performed to unveil the biochemical characteristics of IlpA, and the roles of TLR1 and TLR6 in *V. vulnificus* IlpA-induced activation of human monocytes.

In many known lipoproteins having immunological activity, the portions of the proteins responsible for their immunological activity are located at the N-terminal triacylated lipopeptide region (42, 57, 58). The importance of lipidation in *V. vulnificus* IlpA was also confirmed by an observation that cytokine production in human monocytes triggered by nonlipidated rIlpA was significantly less than that by the intact lipidated rIlpA (Fig. 8). However, the nonlipidated form of *V. vulnificus* IlpA still retains an ability to induce cytokine production in monocytes at high concentrations. This finding raises the possibility that some domain(s) of *V. vulnificus* IlpA protein portion functions as a minor immunostimulant.

One of the distinct pathologies in *V. vulnificus*-causing septicemia is the clinical progression of the systemic inflammatory response (18). The potent immunostimulating activity of IlpA we observed suggests that this microbial factor may be responsible for the progressive pathogenesis of *V. vulnificus*. The importance of surface lipoprotein in systemic inflammation has been already reported in spirochaetal microorganisms lacking LPS (59). Increased levels of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in the serum from *V. vulnificus*-infected patients have been reported (60), which supports the speculated role for dysregulation of the cytokine response for the pathogenesis of *V. vulnificus*. Here we also provide evidence supporting the above clinical observation by showing an increased production of the proinflammatory cytokines, TNF-α and IL-6, in monocytes upon exposure to IlpA of *V. vulnificus*. Thus, *V. vulnificus* IlpA protein, which is capable of activating monocytes to produce cytokines, would be a potential molecule in the development of prevention and a therapy agent for *V. vulnificus* infection.

[3] W. H. Kim and S.-J. Park, unpublished data.

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