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TLR2, but not TLR4, plays a predominant role in the immune responses to cholera vaccines

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ABSTRACT

***Vibrio cholerae* can cause severe diarrhea and dehydration leading to high mortality and morbidity. Current cholera vaccines are formulated with KVC. Although the innate immune responses following vaccination deeply influence the induction of adaptive immunity, the initial recognition of cholera vaccines by the host innate immune system is not well characterized. In this study, the ability of KVC to induce innate immune responses was investigated. Unlike typical Gram-negative bacteria stimulating TLR2 and TLR4, KVC activated TLR2 but hardly TLR4. However, purified *V. cholerae* LPS preferentially stimulated TLR4, although not as potently as LPS of other Gram-negative bacteria, implying that LPS is not a major immunostimulatory component of KVC. Instead, MPFs were similar to KVC in the capacity to activate TLR2, transcription factors, and cytokine expression. Furthermore, OmpU is an abundant membrane protein of *V. cholerae* and could interact with TLR2 for inducing cytokine expression. Notably, cholera vaccine-induced immune responses are impaired in TLR2$^{-/-}$ mice. Conclusively, TLR2 is essential for the immune responses to cholera vaccination, and OmpU is the major immunostimulatory component of cholera vaccines. *J. Leukoc. Biol.* 98: 661–669; 2015.**

Introduction

V. cholerae is a Gram-negative, motile bacterium and the etiologic agent of cholera, provoking severe diarrhea caused by CT in humans [1]. Among more than 200 serogroups identified, only O1 and O139 serogoups have been associated with epidemic cholera [2]. The O1 serogroup of *V. cholerae* is classified into classic and El Tor biotypes, which can be divided further into Inaba, Ogawa, and Hikojima serotypes [3]. Whereas sanitation and hygiene are the best means to prevent cholera, vaccination is still effective, in particular, in developing countries and travelers. At present, 2 oral cholera vaccines are available: Dukoral, formulated with KVC O1 strains containing the rCTB, and Shanchol, with KVC O1 and O139 without rCTB, respectively [4].

Immunization with whole-cell-based vaccines induces innate and adaptive immunity. Innate immunity is triggered in the early phase of immunization by interaction between MAMPs and their cognitive pattern recognition receptors, including TLRs, on and in the host cells [5]. Subsequently, adaptive immunity is induced in the later phase through the recognition of specific antigens, conferring highly effective, antigen-specific immune responses [5]. Recognition through TLRs also influences the subsequent adaptive immune responses, responsible for the immunogenicity of vaccines [6]. For example, TLR4 signaling is essential for the protective immunity induced by a whole-cell pertussis vaccine [7]. TLR4 dependency is mostly a result of LPS, which is found on the outer membrane of Gram-negative bacteria [8]. LPS is an amphiphile consisting of lipid A, core oligosaccharides, and repetitive *O*-PS [9]. The repeating unit in the *O*-PS varies among bacterial species and contributes to structural heterogeneity, whereas the lipid A moiety is highly conserved, exhibiting endotoxic activity, and is responsible for stimulating host innate immunity through TLR4 [10].

Although MAMPs of *V. cholerae*, including LPS, Omps, and the toxin-coregulated pilus, are known to induce protective immune responses [11, 12], little is known about the innate immune

Abbreviations: $^{-/-}$ = deficient, BMM = bone marrow-derived macrophage, CHO = Chinese hamster ovary, CT = cholera toxin, CTB = B subunit of cholera toxin, Hib = *Haemophilus influenzae* type b, HKVC = heat-killed *Vibrio cholerae*, Kdo = 3-deoxy-D-manno-octulosonic acid, KVC = killed *Vibiro cholerae*, MAMP = microbe-associated molecular pattern, MPF = membrane protein fraction, *O*-PS = O-antigen polysaccharide, Omp = outer-membrane protein, Pam3CSK4 = *N*-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine, WT = wild-type

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responses induced by cholera vaccines and their MAMPs. TLR4 has been reported to be necessary for the recognition of *V. cholerae* LPS, so as to induce the production of TNF-α, IL-1β, and MIP-3α via a MyD88-dependent pathway [13]. *V. cholerae* flagellin has been shown to induce IL-1β and IL-8 expression through TLR5-dependent NF-κB and MAPK activation in intestinal epithelial cells [14]. As the initial recognition of and responses to cholera vaccines mediated through TLR have not been well characterized, we investigated how cholera vaccines and KVC stimulated TLRs and what component(s) played an important role in the innate immunity.

MATERIALS AND METHODS

Bacterial strains

V. cholerae El Tor O1 Inaba (T19479), O1 Ogawa (X25049), and O139 (4260B), which were kindly provided by Professor Jan Holmgren (University of Gothenburg, Sweden), and *Shigella flexneri* 5a M90T and *Escherichia coli* BL21 (DE3; Stratagene, La Jolla, CA, USA) were used in this study. All bacterial strains were grown in Luria-Bertani broth at 37°C, 200 rpm, to midlog phase and harvested by centrifugation. Killed bacteria were prepared by incubating cells at 60°C for 1 h or in 0.6% formaldehyde for 5 h with stirring. After washing 3 times with PBS, the cells were resuspended in PBS, followed by determination of CFU/ml based on the OD at 600 nm absorbance. Complete killing of the bacteria was confirmed.

Reagents

Polymyxin B was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pam3CSK4 and *E. coli* LPS were obtained from InvivoGen (San Diego, CA, USA). Anti-human TLR2 (clone TL2.1; IgG2a), anti-human TLR4 (clone HTA125; IgG2a), anti-human CD14 (61D3; IgG1), anti-mouse TLR2 (clone T2.5; IgG1), anti-mouse TLR4 (clone MTS510; IgG2a), and isotype-matched control antibodies (IgG1 and IgG2a) were purchased from eBioscience (San Diego, CA, USA). FITC-labeled anti-human CD25 antibody was obtained from BD Biosciences (San Jose, CA, USA). Shanchol (Shantha Biotechnics, Hyderabad, India) is a bivalent, killed, whole-cell, oral cholera vaccine consisting of O1 and O139 serogroups of formalin KVC or HKVC [15].

Cell culture

Human promonocytic cell line THP-1, mouse macrophage cell line RAW 264.7, and murine lung epithelial cell line LA-4 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured accordingly. WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ Balb/c mice were used to prepare BMMs, as described previously [16]. Heparinized human blood was provided by the Red Cross Korea, and PBMCs were obtained by density gradient centrifugation by use of Ficoll-Plaque Plus. PBMCs were cultured in the RPMI-1640 complete media.

Measurement of TLR2 and TLR4 activation

NF-κB reporter cell lines, CHO/CD14/TLR2 and CHO/CD14/TLR4, were used as described previously [17]. The expression of a membrane-bound reporter protein human CD25 was inducible under the control of NF-κB. CD25 expression was analyzed by flow cytometry (BD Biosciences).

Mice

All animal studies were performed under the approval of the Animal Care and Use Committee of the International Vaccine Institute (Institutional Animal Care and Use Committee Protocol #2012-023). As a mouse pulmonary infection model has been considered a useful model for preclinical evaluation of cholera vaccines [18, 19], 6- to 14-wk-old mice were intranasally immunized with Shanchol at 1×10^9 CFU in 10 μl/mouse twice on d 0 and 14. Sera were obtained from the mice 1 wk after the last immunization.

Preparation of LPS and MPFs from *V. cholerae*

LPS was isolated from *V. cholerae* by use of an LPS extraction kit (Intron, Seongnam, Korea), according to the manufacturer's instructions. The MPFs were prepared by use of ProteoExtract (Calbiochem, San Diego, CA, USA). Proteins were sequentially separated based on their differential detergent solubility into 4 fractions, and the fraction 3 was used as the MPF, as the majority of bacterial membrane proteins was enriched [20]. The MPFs were not contaminated with endotoxins (<5 endotoxin units in the MPFs from 10^7 CFU of *V. cholerae*).

Determination of cytokine expression

A human cytokine array kit (RayBiotech, Norcross, GA, USA) was used for the analysis of secreted cytokine profiles of THP-1 cells. The production of TNF-α and IL-6 was determined by use of ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Determination of antibody responses

V. cholerae-specific LPS antibody titers were determined by ELISA. In brief, 96-well culture plates were coated with 2.5 μg/ml *V. cholerae* LPS and incubated overnight at 4°C. After blocking with 1% BSA in PBS, serially diluted serum samples were added and incubated at room temperature for 2 h. The plates were washed and incubated with alkaline phosphatase-conjugated anti-mouse IgG, anti-mouse IgM, or anti-mouse IgA (Southern Biotechnology, Birmingham, AL, USA) at room temperature for 2 h. The reaction was developed with 4-nitrophenylphosphate for 30 min and read at 405 nm by use of a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Endpoint titer is defined as the reciprocal $\log_2$ of the highest dilution that gives an OD above the cut-off value. The antibody titers below 4 were regarded as 2 for statistical analysis.

EMSA

THP-1 cells were stimulated with HKVC, MPF, or LPS, equivalent to 10^7 CFU/ml *V. cholerae* O1 Inaba or 1 μg/ml *E. coli* LPS for 90 min. Then, nuclear extracts were prepared and subjected to EMSA, as described previously [21].

Identification of OmpU and preparation of rOmpU

The serially extracted fractions from *V. cholerae* were separated on 12.5% gels by SDS-PAGE. The major band at 38 kDa from the fraction 3 was obtained, trypsinized, and subjected to liquid chromatography-coupled electrospray ionization tandem mass spectrometry. The *OmpU* gene was amplified by PCR by use of forward primer (5′-GGCCGCGGATCCGGGACAATAAATTAGGACTT-AATAAGAT-3′) and reverse primer (5′-CCGGGCCTCGAGGAAGTCGT-AACGTAGACCGATA-3′) from genomic DNA of *V. cholerae* O1 Inaba. The PCR product was cloned into the pET21d(+) vector, which contains a 6x-His tag and transformed to *E. coli* BL21(DE3). OmpU protein was expressed under the induction with 1 mM isopropyl β-D-1-thiogalactopyranoside and isolated by use of a Ni-NTA metal affinity chromatography column (Clontech, Mountain View, CA, USA). For some experiments, rOmpU was boiled at 100°C for 30 min or treated with 200 μg/ml proteinase K (Sigma-Aldrich) at 37°C for 1 h, followed by incubation at 75°C for 20 min to inactivate proteinase K. For binding experiments, rOmpU was conjugated with the fluorescent dye Alexa 546 (Invitrogen, Carlsbad, CA, USA), and then unconjugated dye was removed with a PD-10 desalting column (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Statistical analysis

Statistical differences between 2 groups were determined by 2-tailed Student's *t*-test with significance at $P < 0.05$. For comparison of vaccine-induced antibody responses in mice, 1-way ANOVA was carried out, followed by Tukey's multiple comparison, with significance determined at $P < 0.05$. To compare cytokine profiles induced by stimuli by use of cytokine arrays, the fold increase in the expression of cytokines of the MPF or LPS treatment groups was plotted with that of the HKVC treatment group.

Pearson correlation coefficient (r) was obtained to examine the relationship between treatment groups.

RESULTS

TLR2, but not TLR4, mediates cholera vaccine-induced innate immunity

First, we determined whether the cholera vaccines stimulate TLR2 and/or TLR4 by use of 2 reporter cells, CHO/CD14/TLR2 and CHO/CD14/TLR4. Shanchol activated TLR2 and TLR4, but TLR2 activation was 10–100 times higher than TLR4 activation (**Fig. 1A**). As it is known that TLR2 and TLR4 are expressed on THP-1 cells [22], we examined whether Shanchol induces TNF-α production through TLR2 or TLR4 in THP-1 cells. Shanchol induced TNF-α production in THP-1 cells, which was reduced significantly by an antibody blocking TLR2 or CD14 (Fig. 1B). Furthermore, we examined whether Shanchol triggers the mucosal immune responses through TLR2. Treatment with Shanchol significantly induced IL-6 production in murine lung epithelial cell line, LA-4 cells, whereas the IL-6 production was remarkably decreased in the presence of anti-TLR2 antibody but not of anti-TLR4 antibody (Fig. 1C). Concordantly, Shanchol-induced TNF-α production was abrogated substantially by $TLR2^{-/-}$ BMMs, whereas no significant difference was observed in the $TLR4^{-/-}$ BMMs compared with WT BMMs (Fig. 1D). These results suggest that TLR2 is essential for the cholera vaccine-induced innate immunity, but TLR4 is weakly associated with it.

Shanchol is formulated with a mixture of *V. cholerae* O1 Inaba, O1 Ogawa, and O139, inactivated with heat or formaldehyde [15]. TLR2-dependent activation by Shanchol may be a result of an intrinsic property of *V. cholerae* or the inactivation process and/or strain tropism. To test those possibilities, we inactivated various *V. cholerae* strains with heat or formaldehyde. The CD25

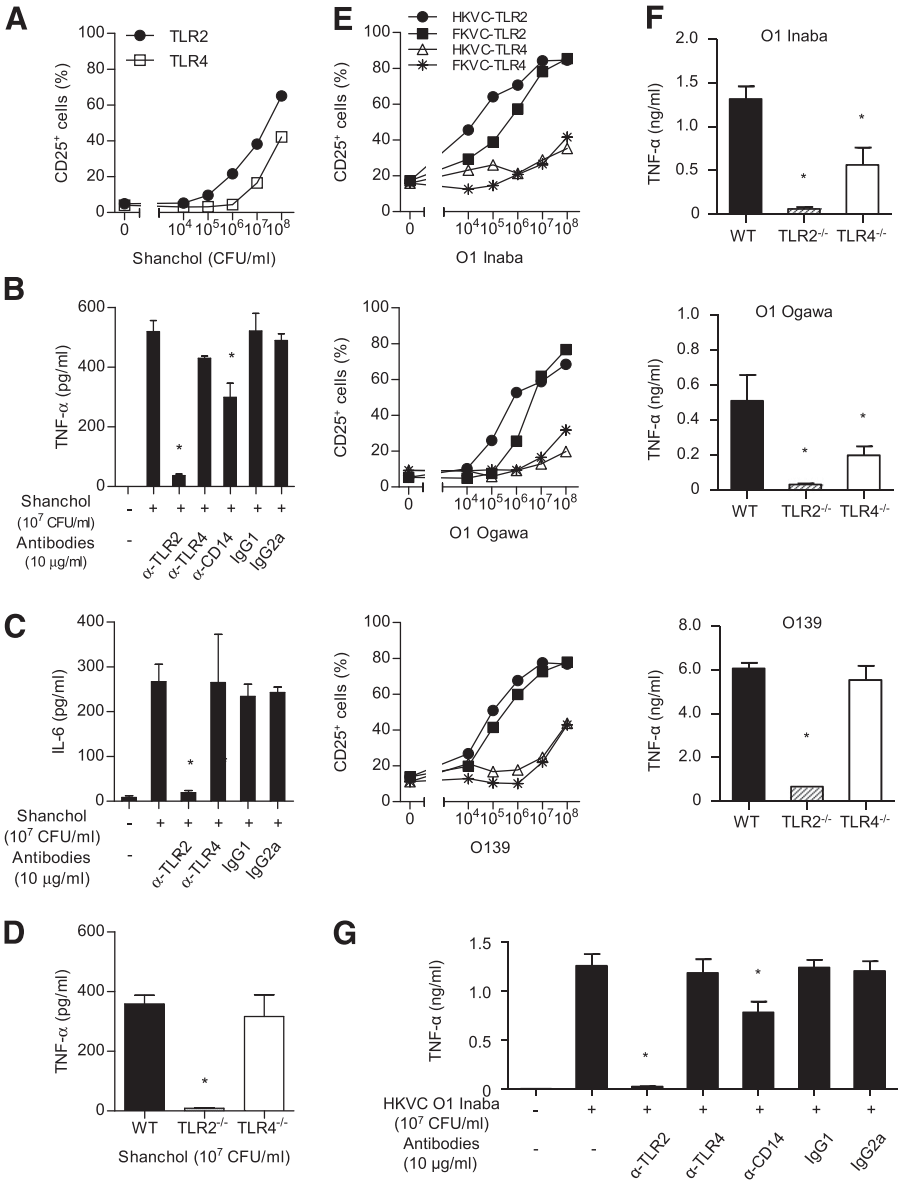

Figure 1. TLR2, not TLR4, is important in the innate immunity induced by KVC. (A) CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were stimulated with the indicated concentrations of Shanchol for 16 h. TLR2- or TLR4-dependent NF-κB activation was then determined by the analysis of CD25 expression via flow cytometric analysis. The data represent 1 of 3 similar experiments. (B) THP-1 cells were pretreated with 10 μg/ml anti-human TLR2, TLR4, and CD14 or their isotype control antibodies for 1 h before stimulation with Shanchol for 6 h. The cell-culture supernatants were then collected, and TNF-α production was determined via ELISA. (C) LA-4 cells were pretreated with 10 μg/ml anti-mouse TLR2 or TLR4 antibodies for 1 h and subsequently stimulated with Shanchol (1×10^7 CFU/ml) for 24 h. The culture supernatants were then collected, and the production of IL-6 was determined by ELISA. (D) BMMs were prepared from WT, $TLR2^{-/-}$, or $TLR4^{-/-}$ mice and subsequently stimulated with Shanchol for 16 h. The cell-culture supernatants were then collected, and TNF-α production was determined by ELISA. (E) CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were stimulated with the indicated concentrations of HKVC or formaldehyde KVC (FKVC) for 16 h. CD25 expression was examined by flow cytometry. The data shown are representative of 3 similar experiments. (F) BMMs from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were stimulated with 1×10^7 CFU/ml HKVC O1 Inaba, O1 Ogawa, and O139 for 16 h. The cell-culture supernatants were then collected, and TNF-α production was determined by ELISA. (G) THP-1 cells were pretreated with 10 μg/ml anti-human TLR2, TLR4, or CD14 antibodies for 1 h and subsequently stimulated with HKVC O1 Inaba for 6 h. IgG1 and IgG2a indicate isotype controls. Values represent means ± SD of triplicates from each group. $^*P < 0.05$ indicates an experimental group significantly different from the control group. One of 3 similar results is shown.

expression was higher in CHO/CD14/TLR2 than in CHO/CD14/TLR4 in response to KVC, regardless of the killing method or *V. cholerae* strain (Fig. 1E). Therefore, HKVC was used in the subsequent experiments. To examine whether TLR2 is necessary for KVC-induced immune response, BMMs derived from TLR2$^{-/-}$, TLR4$^{-/-}$, and WT mice were used. HKVC-induced TNF-α production was abrogated completely in TLR2$^{-/-}$, whereas TLR4$^{-/-}$ produced a similar level of TNF-α as in WT (Fig. 1F). Moreover, TNF-α production was inhibited significantly by anti-TLR2 or anti-CD14 antibody, whereas it was unaffected by anti-TLR4 antibody (Fig. 1G). These results indicate that whole cell-based cholera vaccines preferentially stimulate TLR2 and to a lesser extent TLR4, induce innate immune responses.

V. cholerae is less potent than other enteric Gram-negative bacteria in TLR4 activation

To compare the TLR-stimulating activity of *V. cholerae* with that of other enteric Gram-negative bacteria, CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were stimulated with HKVC, *E. coli*, and *S. flexneri*. CD25 expression by *E. coli* and *S. flexneri* was equally induced through either TLR2 or TLR4, whereas *V. cholerae*-induced CD25 expression was mainly enhanced via TLR2 but not via TLR4 (**Fig. 2**). These results indicate that unlike other Gram-negative bacteria, HKVC preferentially stimulates TLR2, but its TLR4-stimulating activity is weak.

V. cholerae LPS stimulates TLR4 but less potently than other LPSs

As LPS is a major MAMP of Gram-negative bacteria, we hypothesized that preferential stimulation of TLR2 by HKVC could be a result of atypical properties of *V. cholerae* LPS, as is seen in the response to LPS of *Porphyromonsas gingivalis* [23] and *Bacteroides fragilis* [24]. Accordingly, we purified LPS from *V. cholerae* O1 Inaba, O1 Ogawa, and O139 and characterized their TLR-stimulating activities. All LPSs induced CD25 expression only on CHO/CD14/TLR4, not on CHO/CD14/TLR2 cells (**Fig. 3A**).

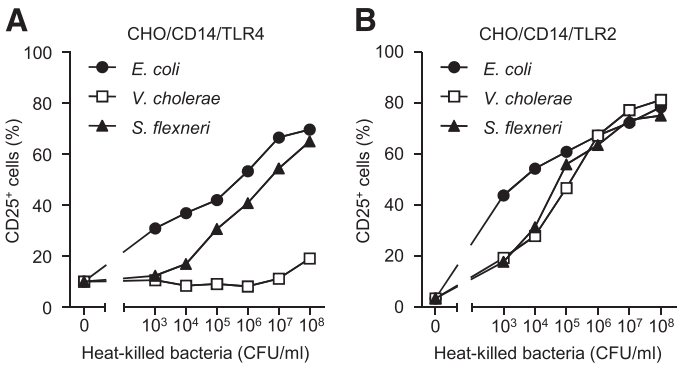

Figure 2. *V. cholerae* is less potent than other enteric Gram-negative bacteria in its TLR4-stimulating ability. CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were stimulated with the indicated concentrations of heat-killed *E. coli*, *S. flexneri*, or *V. cholerae* for 16 h. After stimulation, (A) TLR4- or (B) TLR2-dependent NF-κB activation was determined by CD25 expression via flow cytometric analysis. Values represent the percentage of CD25$^+$ cells of the total CHO/CD14/TLR2 or CHO/CD14/TLR4 cells. The data shown are representative of 3 similar experiments.

Concordantly, all of these LPSs significantly enhanced TNF-α production in BMMs from WT and TLR2$^{-/-}$ mice to the same extent, whereas no induction of TNF-α production was observed in BMMs of TLR4$^{-/-}$ mice (Fig. 3B). Interestingly, *V. cholerae* LPS was much less potent than LPS of *S. flexneri* or *E. coli* with respect to induction of TNF-α production by the mouse macrophage cell line RAW 264.7 (Fig. 3C) or CD25 expression in CHO/CD14/TLR4 cells (Fig. 3D). These results imply that *V. cholerae* LPS is a weak TLR4 stimulator and might not be significantly responsible for the activation of innate immunity by KVC.

The immunostimulatory activity of KVC correlates with that of its MPFs

As our results demonstrate that LPS is not a major immunostimulating MAMP in HKVC, we hypothesized that membrane proteins could play a major role in the innate immunity induced by *V. cholerae*. Thus, we compared immunostimulatory abilities of HKVC, MPF, and LPS by use of human PBMC, THP-1, and mouse BMMs. MPF- and HKVC-induced TNF-α productions were enhanced significantly ($P < 0.01$) to a similar extent compared with the untreated control, whereas LPS had much weaker activity than HKVC or MPF (**Fig. 4A**). Next, we analyzed the protein-expression profiles of 174 human cytokines in THP-1 cells and found that 37 of these cytokines, including TNF-α, were enhanced >2-fold when treated with HKVC (data not shown). The Pearson correlation coefficient comparing the fold increases was $r = 0.721$ between the HKVC- and MPF-treated groups, and $r = 0.059$ between the HKVC- and LPS-stimulated groups (Fig. 4B). Therefore, HKVC-induced cytokine expression correlated better with MPF- than LPS-induced cytokine production. The correlation obtained from the comparative analysis of the cytokine expression profiles was validated by ELISA for the 6 cytokines showing the highest increases in response to HKVC (data not shown). In addition, HKVC and MPF strongly activated NF-κB and AP-1 to bind their cognate DNA elements, whereas LPS had a lesser effect (Fig. 4C). Furthermore, MPF-induced TNF-α production was inhibited by anti-TLR2 and partially by anti-CD14 antibodies but not by anti-TLR4 antibody (Fig. 4D). Collectively, these results suggest that the membrane components better represent the effects of HKVC with respect to immunostimulatory activities.

OmpU, an abundant protein of *V. cholerae*, induces TNF-α production through TLR2

To identify the major immunostimulating component in the MPF, the MPF was separated further by SDS-PAGE. A major protein band was observed at 38 kDa (**Fig. 5A**), and an abundant protein in the band was identified as OmpU. Thus, rOmpU was expressed in *E. coli* and then purified. The rOmpU dose dependently induced TNF-α production in THP-1 cells (Fig. 5B) and CD25 expression in CHO/CD14/TLR2 cells but not in CHO/CD14/TLR4 cells, as seen in response to KVC or MPF (Fig. 5C). Concordantly, antibodies blocking TLR2 or CD14 inhibited the rOmpU-induced TNF-α expression in THP-1 cells, whereas anti-TLR4 antibody did not show such effects (Fig. 5D). To determine whether rOmpU is responsible for the TLR2-stimulating activity, CHO/CD14/TLR2 cells were stimulated with intact, proteinase K-treated or boiled rOmpU. The CD25 expression in CHO/CD14/TLR2 was diminished significantly

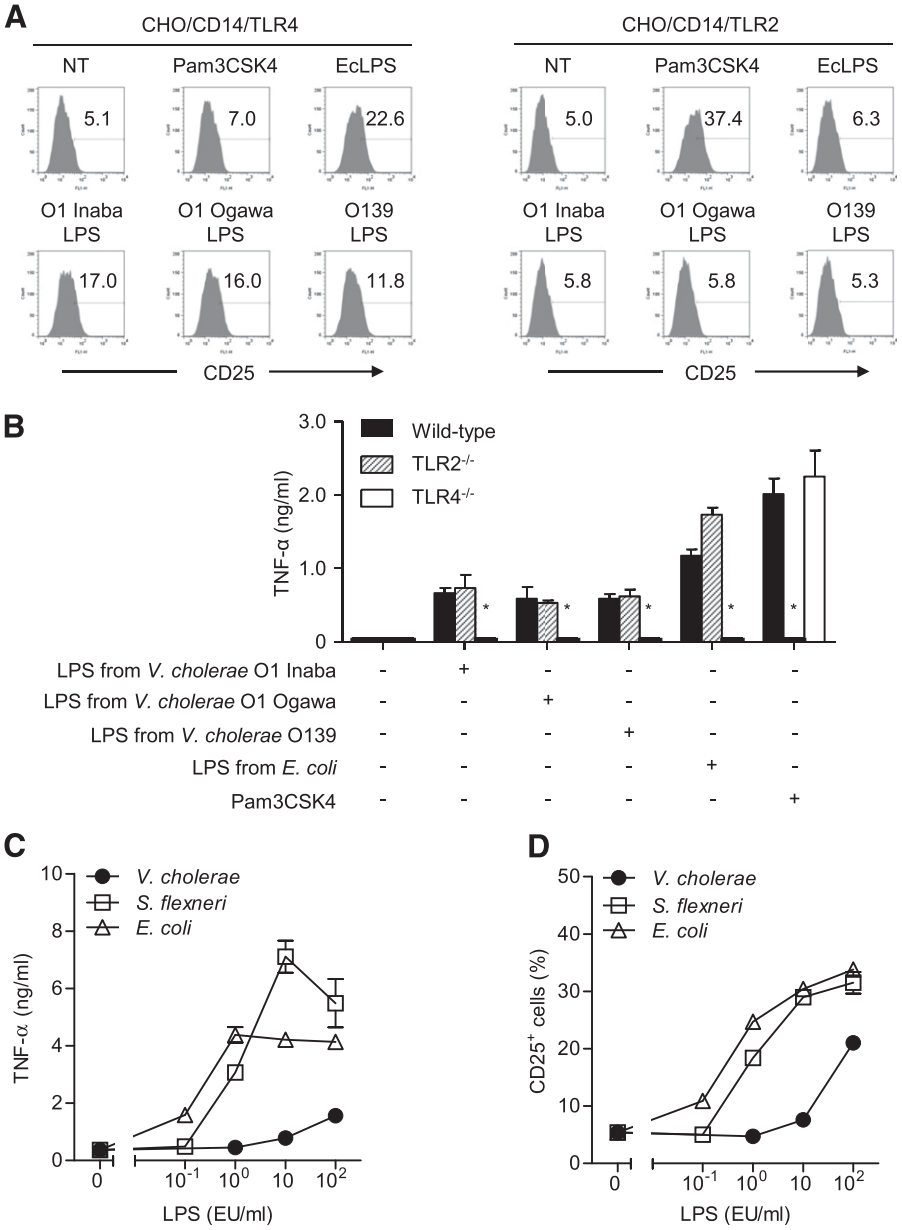

Figure 3. *V. cholerae* LPS stimulates TLR4-like LPS of other bacteria, albeit weakly. (A) CHO/CD14/TLR2 pretreated with 50 μg/ml polymyxin B for 1 h and CHO/CD14/TLR4 cells were non-treated (NT) or treated with Pam3CSK4 (5 μg/ml), *E. coli* LPS (EcLPS; 0.5 μg/ml), or LPS (3×10^3 EU/ml) of *V. cholerae* O1 Inaba, O1 Ogawa, or O139 for 16 h. TLR2- or TLR4-dependent NF-κB activation was then determined by analysis of CD25 expression via flow cytometric analysis. One of 3 similar results is shown. (B) BMMs derived from WT, $TLR2^{-/-}$, or $TLR4^{-/-}$ mice were stimulated with Pam3CSK4 (0.1 μg/ml), *E. coli* LPS (0.1 μg/ml), or the LPS (3×10^3 EU/ml) of *V. cholerae* O1 Inaba, O1 Ogawa, or O139 for 16 h. Cell-culture supernatants were then collected, and TNF-α production was determined via ELISA. Values represent means ± SD of triplicates from each group. $*P < 0.05$ indicates an experimental group significantly different from the control group. One of 3 similar results is shown. (C) RAW 264.7 cells were stimulated with the indicated concentration of LPS from *V. cholerae*, *S. flexneri*, or *E. coli* for 16 h, and the level of TNF-α in the cell-culture media was determined by ELISA. Values represent means ± SD of triplicates from each group. (D) CHO/CD14/TLR4 cells were treated with the indicated concentration of LPS from *V. cholerae*, *S. flexneri*, or *E. coli* for 16 h. TLR2- or TLR4-dependent NF-κB activation was then determined by analysis of CD25 expression via flow cytometric analysis. Values represent means ± SD of triplicates from each group. One of 3 similar results is shown.

upon exposure to proteinase K-treated rOmpU or boiled rOmpU (Fig. 5E), implying that the TLR2-stimulating activity was not a result of heat-stable impurities, such as immunologically active lipids or polysaccharides. To examine further the interaction of rOmpU with TLR2, BMMs derived from WT or $TLR2^{-/-}$ mice were mixed with Alexa 546-labeled rOmpU, which preferentially bound to WT BMMs but barely bound to $TLR2^{-/-}$ BMMs, and proteinase K treatment or boiling at 100°C abrogated the ability of Alexa 546-labeled OmpU to bind to WT BMMs (Fig. 5F). Collectively, these results indicate that OmpU is a major membrane protein of *V. cholerae* that induces immunostimulation through TLR2.

Cholera vaccine-induced immune responses are impaired in $TLR2^{-/-}$ mice

As TLR2 appears to be a key receptor to elicit the innate immune response to KVC, we hypothesized that the TLR2-mediated immune response would be critical for the immunogenicity of cholera vaccines. To examine the role of TLR2 in the generation of antigen-specific antibodies, we immunized WT and $TLR2^{-/-}$ mice intranasally with Shanchol. Anti-LPS-specific IgG and IgM, but not IgA, were highly increased ($P < 0.05$) in the sera of WT mice when compared with the control group (**Fig. 6**). Notably, anti-LPS IgG and IgM levels were decreased significantly ($P < 0.05$) in $TLR2^{-/-}$ mice when compared with those in WT. In contrast, the levels of anti-LPS-specific IgG, IgM, and IgA were not decreased significantly in $TLR4^{-/-}$ mice compared with those in WT. These results suggest that the innate immunity through TLR2 is essential for the immunogenicity of cholera vaccines.

DISCUSSION

Numerous studies have been reported on the adaptive immune responses induced by killed, whole-cell cholera vaccines, yet the

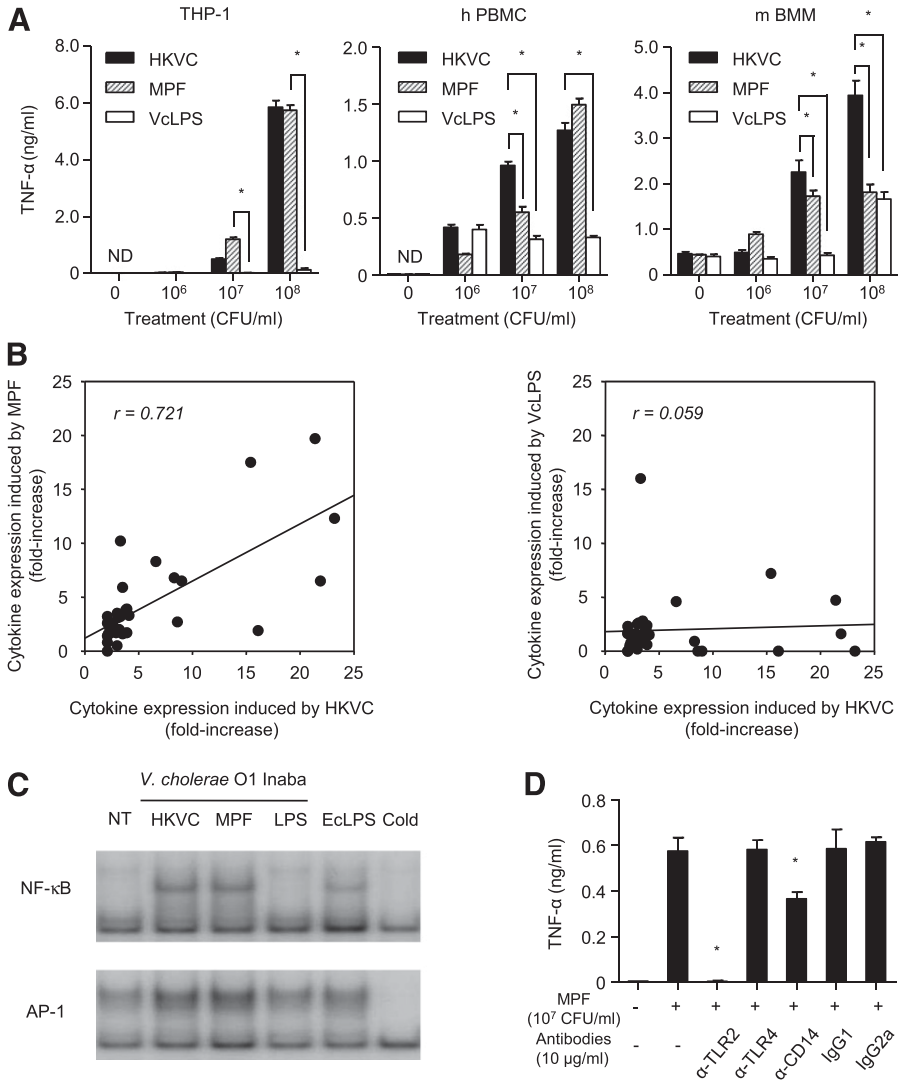

Figure 4. The immunostimulatory activity of KVC correlates with that of its MPFs but not with its LPS. (A) THP-1 cells, human (h) PBMCs, and mouse (m) BMMs were stimulated with the indicated concentration of HKVC O1 Inaba, the MPF, or LPS [*V. cholerae* LPS (VcLPS)] for 6 h (for THP-1 cells) or 16 h (for the other cell types). Cell-culture supernatants were then collected for analysis of TNF-α production by ELISA. One of 3 similar results is shown. ND, Not detected. (B) THP-1 cells were treated with HKVC at 1×10^7 CFU/ml or the comparable amounts of MPF and LPS for 16 h, and cell-culture supernatants were then analyzed for protein profiling by use of a cytokine expression array. A >2-fold increase in the expression of cytokines by HKVC over nontreated control was compared with increases induced by the MPF or LPS. Diagonal lines indicate regression of fold increase in the expression of cytokines induced by each stimulus. Pearson correlation coefficient (r) was obtained to examine the correlation between treatments. (C) THP-1 cells were stimulated as described above for 90 min. Nuclear extract was prepared and incubated with [^{32}P]-labeled oligonucleotides containing the consensus-binding sequences of NF-κB or AP-1. Protein-DNA binding complexes were electrophoresed and visualized by autoradiography. One picomole of unlabeled probe (marked as "Cold") was used for the competition assay. Results represent 1 of 3 similar experiments. (D) THP-1 cells were pretreated with 10 μg/ml anti-human TLR2, TLR4, or CD14 antibodies or the isotype controls (IgG1 and IgG2a) for 1 h and subsequently stimulated with the MPF isolated from *V. cholerae* O1 Inaba for an additional 6 h. The level of TNF-α in the cell-culture supernatants was then determined via ELISA. Values represent means ± SD of triplicates from each group. *$P < 0.05$ indicates an experimental group significantly different from the control group. Results represent 1 of 3 similar experiments.

innate immune responses elicited at the early stages of immunization are poorly understood. In the present study, we firstly demonstrated that unlike other enteric Gram-negative bacteria, KVC weakly stimulated TLR4 but substantially activated TLR2. The immunostimulatory capacity of the MPF, rather than LPS, better correlated with that of killed, whole-cell *V. cholerae*. Notably, *V. cholerae* OmpU demonstrated a potent, immunostimulatory activity through TLR2. Furthermore, TLR2 is critical for the induction of an antigen-specific antibody response to cholera vaccination. The results suggest that OmpU might be important in cholera vaccine-induced innate immune responses, and TLR2 is essential for innate and adaptive immunity against *V. cholerae*.

Given the fact that a large number of enteric Gram-negative bacteria are recognized by TLR4 and TLR2 [5, 25], the capacity of *V. cholerae* to activate TLR2, but not TLR4, preferentially is unusual. However, it is not the only case of a unique feature to *V. cholerae*. For instance, heat-killed *Brucella abortus* stimulates macrophages to produce proinflammatory cytokines, and this is inhibited by TLR2 blocking but is unaffected by polymyxin B or TLR4 blocking [26]. In addition, whole bacteria of *Helicobacter spp.* induced immune responses via TLR2, whereas LPS of the bacteria was found to be a TLR4 ligand with weak immunostimulatory activity [25].

Our initial hypothesis was that *V. cholerae* LPS could induce atypical activation of TLR2 similar to that seen for *P. gingivalis* [23] and *B. fragilis* [24]. However, *V. cholerae* LPS stimulated TLR4, as LPS of most Gram-negative bacteria [13], even though it is very weak in its immunostimulation. In agreement with our results, *V. cholerae* LPS showed 1000 times lower activity than LPS of other bacteria to induce cytokine production [27]. This weak activity might be associated with its structural features. Firstly, *V. cholerae* contains lipid A with a single Kdo moiety, whereas others, including *E. coli* LPS, have 2 Kdo moieties [28]. Remarkably, synthetic lipid A with a single or no Kdo unit has a decreased cytokine-inducing activity [29]. Secondly, *V. cholerae* has phosphorylated Kdo, unlike other bacteria that possess unphosphorylated Kdo [28]. Indeed, dephosphorylation of Kdo in the

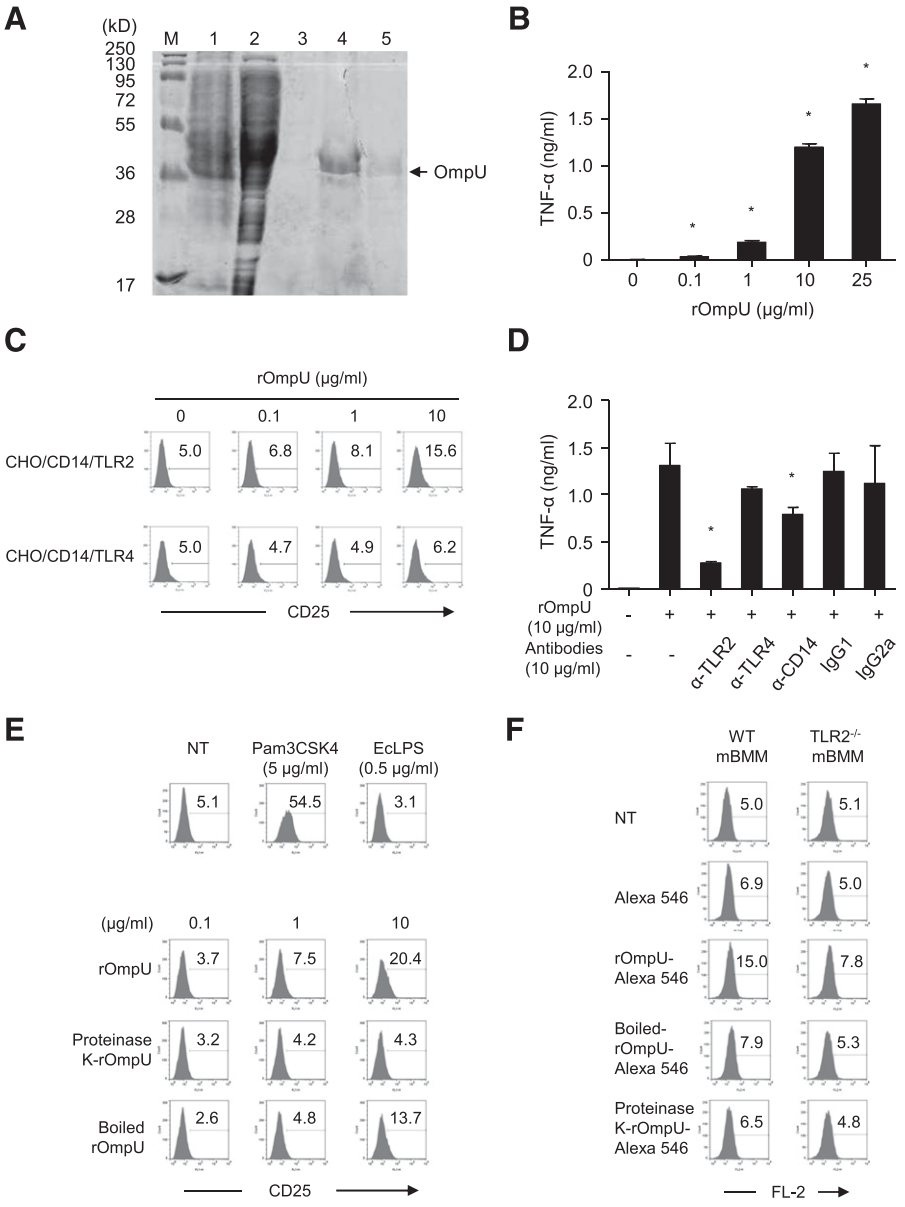

Figure 5. OmpU induces TNF-α production via an interaction with TLR2. (A) Protein profiles of *V. cholerae* O1 Inaba were analyzed by use of 12.5% SDS-PAGE and visualized by Coomassie blue staining. MPFs were sequentially isolated based on their detergent solubility. M, Size marker; Lane 1, HKVC; Lanes 2–5, MPF 1–4, respectively, by differential SDS solubility. (B) THP-1 cells were stimulated with rOmpU (0–25 μg/ml) in the presence of 50 μg/ml polymyxin B for 6 h. Cell-culture supernatants were then collected, and TNF-α production was determined by ELISA. (C) CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were treated with rOmpU (0–10 μg/ml) for 16 h. TLR2- or TLR4-dependent NF-κB activation was then determined by analysis of CD25 expression via flow cytometric analysis. The results represent 1 of 3 similar experiments. (D) THP-1 cells were pretreated with 10 μg/ml anti-human TLR2, TLR4, or CD14 antibodies in the presence of polymyxin B at 50 μg/ml for 1 h, followed by stimulation with 10 μg/ml rOmpU for an additional 6 h. The level of TNF-α in the cell-culture supernatants was then determined via ELISA. Values represent means ± SD of triplicates from each group. $*P < 0.05$ indicates an experimental group significantly different from the control group. One of 3 similar results is shown. (E) CHO/CD14/TLR2 cells were preincubated with 50 μg/ml polymyxin B for 1 h, followed by treatment with rOmpU, proteinase K-treated rOmpU, boiled rOmpU, Pam3CSK4, or *E. coli* LPS at the indicated concentrations for 16 h. TLR2-dependent NF-κB activation was then determined by analysis of CD25 expression via flow cytometric analysis. The results represent 1 of 3 similar experiments. (F) BMMs were prepared from WT and TLR2$^{-/-}$ Balb/c mice and pre-incubated with 50 μg/ml polymyxin B for 1 h. Cells were then stimulated with 10 μg/ml rOmpU, boiled rOmpU, or proteinase K-treated rOmpU labeled with Alexa 546 dye for 1 h, and the binding of rOmpU was subsequently determined by flow cytometry. The results represent 1 of 3 similar experiments.

lipid A of *V. cholerae* enhanced the capability of LPS to induce IL-1 production in human monocytes [30]. Thirdly, the ratio of phosphates to amino groups of *V. cholerae* LPS is 2.4–3.0 times lower than that of *E. coli* LPS [31]. Partial dephosphorylation of LPS that uses alkaline phosphatase resulted in a decreased induction of TNF-α expression [32]. As LPS has been reported to interact with its receptors by electrostatic force [33], *V. cholerae* LPS, with its lower negative charge, as a result of its single Kdo and fewer phosphate groups, may provide a weaker interaction with the cationic LPS-binding protein, leading to decreased LPS signaling.

V. cholerae OmpU is a porin protein that forms homotrimers of 38 kDa monomers. It comprises 30–60% of the total Omps of *V. cholerae* and functions as a channel for the transport of hydrophilic solutes [34]. Given the fact that OmpU was identified as an abundant protein in the MPF with immunostimulatory potential through TLR2 in whole-cell *V. cholerae*, this protein most likely plays a central role in triggering inflammatory responses. Indeed, OmpU induced the expression of MCP-1 and IL-6 in intestinal epithelial cells stimulated with *V. cholerae* [35], and it enhanced production of NO, TNF-α, and IL-6 in RAW 264.7 and THP-1 cells and human PBMCs [36]. In agreement with our results, Omps of Gram-negative bacteria play an important role in the induction of inflammatory cytokines through TLR2-dependent signaling, as has been demonstrated in *B. abortus* [26], Hib [37], and *Klebsiella pneumoniae* [38].

Notably, TLR2$^{-/-}$ mice produced much less LPS-specific IgG and IgM antibodies after cholera vaccination than those in WT. In agreement with our results, immunization of TLR2$^{-/-}$ mice with the outer-surface lipoprotein of *Borrelia burgdorferi* [39] and Hib-meningococcal Omp complex [37] showed impaired antibody responses. It is likely that insufficient induction of cytokines,

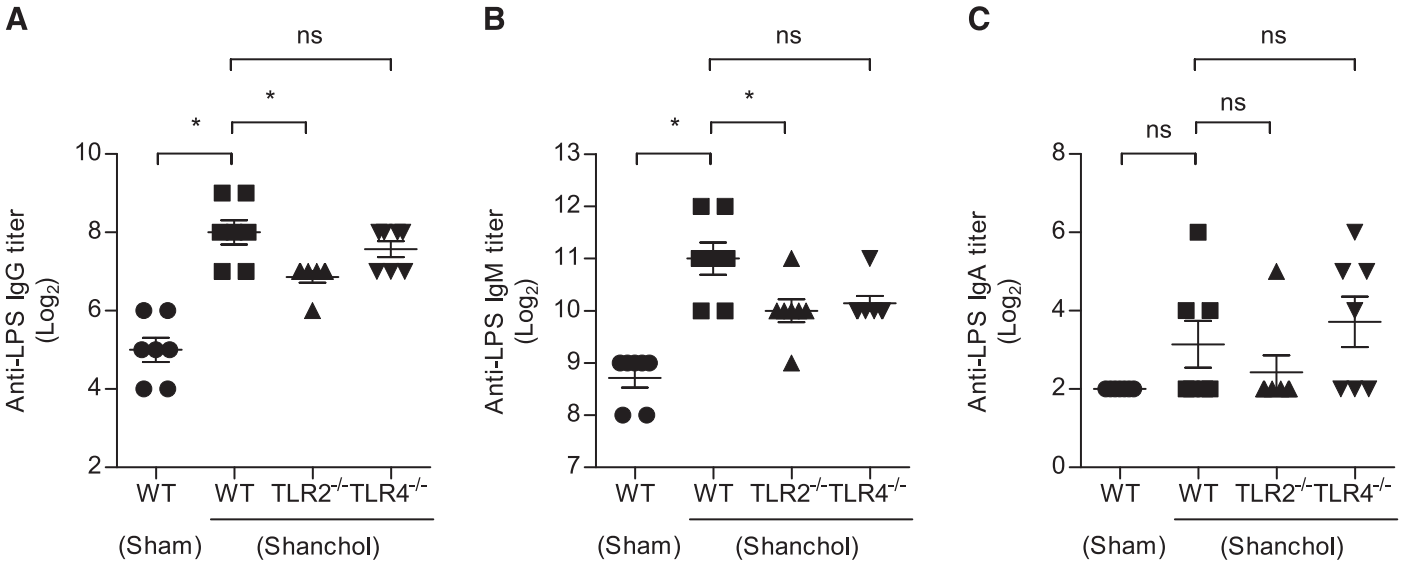

Figure 6. TLR2 is essential for cholera vaccine-induced antibody responses. WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice ($n = 7$) were intranasally immunized twice with Shanchol on d 0 and 14. Serum samples were obtained at 1 wk after the last immunization, and then *V. cholerae* LPS-specific IgG (A), IgM (B), and IgA (C) titers were measured. Data represent mean values ± SEM from each group and were obtained from 2 independent experiments. *$P < 0.05$ indicates that the TLR2$^{-/-}$ mice group is significantly different from the WT group immunized with cholera vaccine by use of 1-way ANOVA with Tukey's multiple comparison test. ns, not significant.

such as TNF-α and IL-6 in macrophages from TLR2$^{-/-}$ mice, may not fully induce activation and differentiation of T_H and B cells, resulting in impaired antibody production [37, 39]. Furthermore, MyD88 is an essential intracellular messenger for TLR2 signaling [40], and MyD88$^{-/-}$ mice immunized with bacillus Calmette-Guérin have not been protected from infection with *Mycobacterium tuberculosis* [41]. Engagement of TLR signaling through MyD88 seems to be a prerequisite for the induction of appropriate antibody responses by controlling B cells at multiple stages [42]. A previous report has shown that *Neisseria meningitidis* OmpC exerts adjuvant-like activity for the activation of macrophages and increases the expression of B7-2, a B cell costimulator, through TLR2 signaling [37]. Our study indicated that increased *V. cholerae* OmpU-specific antibody responses were not observed in sera from WT mice administered with Shanchol (data not shown), assuming that OmpU may act like an adjuvant to enhance humoral immune responses by triggering the innate immunity via TLR2.

In summary, this study demonstrates that TLR2 is the major mediator of the innate immunity induced by cholera vaccine. Furthermore, the membrane protein OmpU plays an important role in *V. cholerae*-induced innate immunity. Given the fact that the innate immunity established at the initial phase of vaccination is a critical event for the determination of adaptive immune responses, our results provide an important insight into the immune responses to current cholera vaccines, providing better understanding of the mechanisms and characteristics of vaccine-induced immunity. Further studies will be needed to examine whether OmpU interacts with TLR2 in a similar way as do lipopeptides and lipoteichoic acid [43] and how OmpU affects adaptive immunity in response to cholera vaccines. Remarkably, anti-OmpU sera showed passive, protective immunity against homologous strains, as well as heterologous *V. cholerae* O1 Ogawa and O139 in an animal model [11]. Therefore, OmpU appears to be crucial for the induction of innate immune responses conferred by *V. cholerae* whole-cell vaccines.

AUTHORSHIP

S.H.H. conceived of the idea. J.S.Y., H.J.K., and S.H.H. designed the experiments. J.S.Y., H.J.K., S.-S.K., and K.W.K. performed experiments. J.S.Y., H.J.K., S.-S.K., K.W.K., and S.H.H. analyzed the data. D.W.K., C.-H.Y., S.-J.P., H.S.S., and B.B.F. provided critical comments. All authors contributed to the interpretation of the results, followed by writing and/or reviewing the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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