

# The FomA Porin from *Fusobacterium nucleatum* Is a Toll-Like Receptor 2 Agonist with Immune Adjuvant Activity

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Many bacterial components selectively activate immune and nonhematopoietic target cells via Toll-like receptor (TLR) signaling; modulation of such host responses defines the immune adjuvant properties of these bacterial products. For example, the outer membrane protein porins from *Neisseria*, *Salmonella*, and *Shigella* are known TLR2 agonists with established systemic and mucosal immune adjuvanticity. Early work indicated that the FomA porin from *Fusobacterium nucleatum* has immune adjuvant activity in mice. Using a purified recombinant FomA, we have verified its immune stimulatory properties and have defined a role for TLR2 signaling in its *in vitro* and *in vivo* activity. FomA induces interleukin 8 (IL-8) secretion and NF- $\kappa$ B-dependent luciferase activity in HEK cells expressing TLR2, IL-6 secretion, and cell surface upregulation of CD86 and major histocompatibility complex (MHC) II in primary B cells from wild-type mice, but it fails to activate cells from TLR2 knockout mice. Accordingly, the immune adjuvant activity of FomA is also TLR2 dependent. In a mouse model of immunization with ovalbumin (OVA), FomA induces enhanced production of OVA-specific IgM and IgG, including IgG1 and IgG2b antibodies, as well as enhanced secretion of IL-10 and IL-6, consistent with a Th2-type adjuvant effect. We also observe a moderate production of anti-FomA antibodies, suggesting that FomA is also immunogenic, a quality that is also TLR2 dependent. Therefore, modulation of host immune responses by FomA may be effective for targeting general host immunity not only to pathogens (as a novel TLR2 adjuvant) but also to *F. nucleatum* itself (as an antigen), expanding its use as a self-adjuvanted antigen in an immunization strategy against polymicrobial infections, including those by *F. nucleatum*.

In the past 2 decades, modulation of host innate and adaptive immunity by bacterial products has been explained *in vitro* and *in vivo* by signaling via Toll-like receptors (TLRs), cell surface and intracellular receptors that recognize microbial products (pathogen-associated molecular patterns [PAMPs]) (1, 48). Upon TLR engagement, activation of NF- $\kappa$ B nuclear translocation and mitogen-activated protein (MAP) kinases leads to secretion of inflammatory mediators, expression of costimulatory ligands, and major histocompatibility complex (MHC) molecules, ultimately enhancing host humoral and cellular immune response. Numerous bacterial components have been shown to activate diverse TLRs; for example, lipopolysaccharide (LPS) is the ligand for TLR4 (in complex with MD2) (15, 60), flagellin activates TLR5 (25), and CpG DNA engages TLR9 (7). TLR2 has the broadest ligand repertoire, due to its hetero-dimerization with TLR1 or TLR6; these different TLR2 complexes recognize lipopeptides (19, 31), peptidoglycans (4), and porins from numerous microorganisms (13, 21, 45, 47, 57). Immune cell activation via TLR signaling is the basis for the observed immune adjuvant effect of these bacterial components (30). Among the known bacterial TLR2 ligands with adjuvant activity, the immune stimulatory effect of neisserial porin PorB (14, 20, 41, 65) and porins from *Shigella*, *Salmonella*, and *Haemophilus influenzae* (2, 56, 57), as well as the *Escherichia coli* heat-labile enterotoxin B subunits [LT-IIa-B<sub>(5)</sub> and LT-IIb-B<sub>(5)</sub>], has been extensively characterized (24, 39).

FomA (~41 kDa) is a major outer membrane protein from *Fusobacterium nucleatum*, a human oral pathogen (9). It exhibits the functional properties of a general trimeric diffusion porin, although it has no sequence similarity to other porins (6, 10). According to the proposed porin topology model, most porin monomers have a  $\beta$ -barrel conformation and 16 transmembrane antiparallel  $\beta$ -strands (64). The  $\beta$ -strands are connected by short loops at the periplasmic side, while longer loops are exposed to the

outside with eight surface-exposed loops. The third surface-exposed loop from the N terminus (loop 3) of most porins, the eyelet loop, folds back into the pore lumen and plays a major role in the permeability of the porin. In contrast, FomA monomers have a 14-strand  $\beta$ -barrel fold with six surface-exposed, variable hydrophilic loops (53) and loop 6 actually functions as the eyelet loop (37, 54). Both native FomA and recombinant FomA expressed in *E. coli* have been purified (18, 23, 28, 52). Proper refolding of recombinant FomA has been demonstrated, and its structural and functional features have been extensively described, including trimer formation, insertion in lipid bilayers, and pore-forming functions (3, 35, 36, 52).

Previous studies in the late 1980s, antecedent to identification of TLRs, reported immune adjuvant activity of FomA in a mouse model of immunization with sheep red blood cells (SRBCs) (62). This work suggested that the effect of purified native FomA was independent of an abundant *F. nucleatum* LPS contamination of such preparation. So far, this assumption has not been validated in the context of TLR-dependent signaling induced by different bacterial products. Other *in vitro* effects attributed to FomA included induction of polyclonal B cell activation and mitogenicity of murine splenocytes, stimulation of guinea pig peritoneal macrophages, and enhancement of human blood monocyte migration (62). More recently, indirect evidence has suggested a role for

Received 19 April 2012 Returned for modification 8 May 2012

Accepted 16 May 2012

Published ahead of print 23 May 2012

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doi:10.1128/CVI.00236-12

TLR2 in the activity of FomA. For example, FomA-containing bacterial sonicates induced TLR2-dependent interleukin 8 (IL-8) production (27, 33) but failed to induce reactive-oxygen species (ROS) production by macrophages from TLR2 knockout (KO) mice compared to LPS-sensitive and LPS-nonresponder mice (43). FomA-containing outer membrane preparations also induced expression of antimicrobial compounds, human  $\beta$ -defensins (hBD-2 and hBD-3), in human gingival epithelial cells via TLR2-dependent MAPK/p38 and MAPK/JNK signaling. This was prevented by heat treatment of *F. nucleatum* and was not due to *Fusobacterium* LPS (29, 38, 51).

While no further studies on the immune stimulatory effect of FomA have been carried out to date, evidence of its immunogenicity and of the protective effect of anti-FomA antibodies on *F. nucleatum* infection has been reported. For example, immunization of mice with whole FomA-expressing *E. coli* organisms or with recombinant FomA and the adjuvant cholera toxin (CT) (a non-TLR adjuvant [26]) induced high levels of circulating and salivary anti-FomA IgG and IgA antibodies protective against *F. nucleatum* infection (40, 50).

Based on the structural and functional similarities between FomA and other bacterial porins with well-established TLR2-dependent immune stimulatory properties, in this work the potential TLR2 adjuvant nature of FomA was reevaluated. Here, the molecular mechanism of cell activation by purified, LPS-free FomA is examined *in vitro* and its immune adjuvant activity is evaluated *in vivo* in a mouse model of immunization with the prototype antigen, ovalbumin (OVA).

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* strain PC2889 harboring the plasmid pET-10953 expressing recombinant FomA protein (52) was a kind gift from H. Kleinschmidt (University of Konstanz, Germany). The organisms were grown aerobically for 12 to 15 h at 37°C in LB medium supplemented with 100  $\mu$ g/ml ampicillin. Expression of FomA was induced with 0.1 M IPTG for an additional 3 h. The cells were harvested by centrifugation at 4°C, and FomA was purified from inclusion bodies as described below.

**FomA purification.** Recombinant FomA was purified by adapting the methods described for neisserial PorB purification (46, 55). FomA-containing *E. coli* inclusion bodies were suspended in 8 M urea and 5% Zwittergent 3-14 to extract the total protein content and were subjected to protein chromatography purification (46). First, a DEAE-Sepharose column was used and FomA was eluted with buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, 0.05% Zwittergent 3-14, 0.02% Na<sub>3</sub>N, and 0.2 M NaCl. The FomA-containing fractions, identified by SDS-PAGE and Coomassie staining, were precipitated with 80% (vol/vol) ethanol and separated by size exclusion chromatography on a Sephacryl-300 column with 100 mM Tris, pH 8.0, 10 mM EDTA, 0.2 M NaCl, 0.05% Zwittergent 3-14, and 0.02% Na<sub>3</sub>N (46). FomA-enriched fractions, identified and pooled as described above, were concentrated and further separated on a Matrex Cellufine sulfate column eluted with a 0.2 M to 0.5 M NaCl gradient in 50 mM Tris, pH 7.5, 10 mM EDTA, 0.05% Zwittergent 3-14, and 0.02% Na<sub>3</sub>N. Concentrated purified FomA fractions were resuspended in 10% D-octyl-glucoside (DOG) in 10 mM HEPES, pH 7.5, followed by extensive dialysis in phosphate-buffered saline (PBS) containing 0.02% Na<sub>3</sub>N for formation of detergent-free protein micelles (proteosomes). The protein concentration was determined using the bicinchoninic acid (BCA) assay.

**Gel electrophoresis and Western blotting.** Equal amounts of protein fractions were analyzed by Coomassie brilliant blue staining with 12% SDS-PAGE or silver staining with 15% SDS-PAGE. For silver staining, 2  $\mu$ g total of purified FomA and of *E. coli* LPS, as a positive control, was used. Prior to electrophoresis, the samples were dissolved in 4 $\times$  sample

buffer (400 mM dithiothreitol [DTT], 40% glycerol, 8% SDS, 200 mM Tris, pH 6.8) and heated for 5 min at 100°C. Western blot analyses were performed using 1  $\mu$ g total of purified FomA. Blots were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline (TBS), pH 7.6, 0.1% Tween 20 (TBS-T) and incubated with sera from mice immunized with FomA as described below (1:1,000 dilution) in 5% bovine serum albumin (BSA) in TBS-T overnight at 4°C followed by horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody (Cell Signaling). The immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Amersham).

**Cell culture and stimulation.** Stably transfected HEK cells overexpressing TLR2 and TLR4 or an empty vector (pcDNA) (15, 47) were cultured in Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 10  $\mu$ g/ml ciprofloxacin. Murine B cells were isolated from spleens of C57BL/6 mice and TLR2 knockout mice as previously described (66) and grown in RPMI (Mediatech) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM HEPES, and 10 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME). HEK cells (10<sup>5</sup> cells/ml) and B cells (10<sup>6</sup> cells/ml) were incubated with purified FomA (10  $\mu$ g/ml), Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml) (InvivoGen), lipoprotein-free *E. coli* LPS (100 ng/ml) (Sigma), or recombinant human tumor necrosis factor alpha (TNF- $\alpha$ ) (20 ng/ml) (eBioscience) in triplicate wells for 18 h at 37°C.

**NF- $\kappa$ B luciferase reporter assay.** HEK cells were transiently transfected with an NF- $\kappa$ B luciferase reporter vector as previously described (15, 47). Transfected cells were left to adhere overnight in 24-well cell culture plates and stimulated the next day as described above. Luciferase activity was measured using commercial reagents (Promega) per the manufacturer's protocol, and luminescence was assessed using a Wallac Victor2 luminometer.

**Cytokine ELISA.** IL-8 and IL-6 secretion was measured by enzyme-linked immunosorbent assay (ELISA) of supernatants from HEK cell cultures and murine B cell cultures stimulated as described above using Opt-EIA kits (BD Biosciences) per the manufacturer's protocol. Results are expressed as pg/ml  $\pm$  standard deviation (SD). Gamma interferon (IFN- $\gamma$ ), IL-10, IL-6 (R&D System ELISA kit), and TNF- $\alpha$  (eBiosciences ELISA kit) were measured by ELISA of sera from mice immunized as described below. Preimmune sera from each group of mice were pooled and used as a negative control. Results are expressed as pg/ml normalized to preimmune sera  $\pm$  SD.

**Flow cytometry.** Purified mouse B lymphocytes (10<sup>6</sup>/ml) stimulated as described above were collected and examined for upregulation of cell surface activation markers using anti-CD86 and anti MHC-class II murine fluorescein isothiocyanate (FITC)-labeled antibodies (PharMingen, San Diego, CA) (45). As a negative control, anti-rat IgG FITC-labeled antibody was used. Labeled cells were analyzed by flow cytometry on a FACScan flow cytometer using CellQuest acquisition and analysis software (Becton, Dickinson, Mountain View, CA). Gating was used to exclude cellular debris. All the histograms shown are representative of three separate experiments.

**Immunization of animals.** Animals were housed and cared for in accordance with National Institutes of Health (NIH) and Boston University protocols for the care and use of laboratory animals. Six-week-old female C57BL/6 mice (Jackson Laboratory) and TLR2 knockout mice (63) ( $n = 4$ ) were immunized subcutaneously three times at 2-week intervals with 10  $\mu$ g of ovalbumin (OVA) combined with either 10  $\mu$ g of FomA in a 1:1 mixture (based on previous experience with neisserial PorB [41, 42]) or 200  $\mu$ g of alum (Sigma) as adjuvants in a total volume of 100  $\mu$ l of sterile PBS. Additional groups were immunized with OVA alone and with FomA alone in the absence of other adjuvants. Preimmune sera were collected prior to the first immunization and 2 weeks after each subsequent immunization (1st bleed at week 2, 2nd bleed at week 4, and 3rd bleed at week 6). Sera were aliquoted and kept at -20°C until further analysis. Ovalbumin was obtained from commercial chicken egg whites by freeze-drying followed by lyophilization and resuspension of the total

egg white proteins in sterile PBS (61). Ovalbumin obtained with this method represents ~65% of the total protein content and is LPS-free.

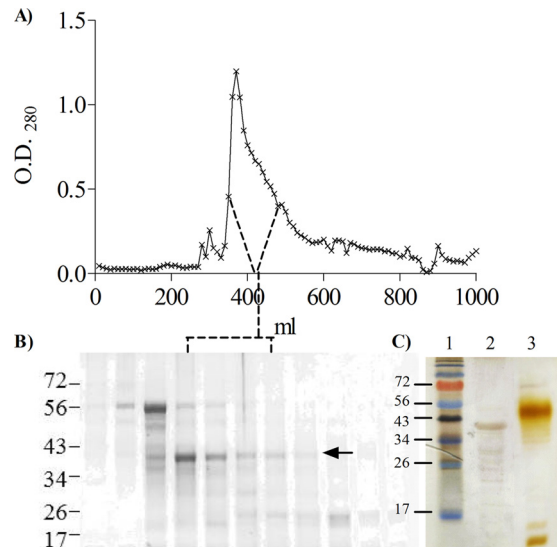
**Serum antibody ELISA.** Levels of OVA-specific total IgG and IgM in the sera of immunized mice were determined by ELISA. Immulon 96-well plates were coated overnight with 5  $\mu$ g of OVA in carbonate buffer, pH 9.6, at 4°C, washed three times with PBS-0.05% Tween 20, and blocked for 2 h with 5% BSA in PBS at room temperature. Serial dilutions of individual mouse sera were added, and the plates were incubated at 4°C for 24 h. Plates were washed and incubated with secondary anti-mouse IgG or IgM alkaline phosphatase (AP)-conjugated antibodies (Sigma) for 2 h at room temperature followed by detection with 1-step PNPP substrate (Pierce) as specified by the manufacturer. Anti-FomA IgG and IgM antibodies were measured as described above using plates coated with 2  $\mu$ g/ml of purified FomA. The absorbance was measured as the optical density at 405 nm (OD<sub>405</sub>), and antibody quantification was performed using IgG and IgM standard curves (41). Results are expressed in  $\mu$ g/ml of specific antibodies. For detection of IgG antibody subclasses to OVA and to FomA, plates were coated as described above and incubated with mouse sera (1:100 dilution) followed by goat anti-mouse specific anti-IgG1, -IgG2b, and -IgG2c AP-conjugated secondary antibodies (Southern Biotech). Results are expressed as anti-OVA- and anti-FomA-specific IgG subclass titers at OD<sub>405</sub> normalized to those from the preimmune groups.

**Statistical analysis.** Statistical analyses were calculated using GraphPad PRISM software. *P* values were calculated by unpaired *t* test and by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison posttest, with 99.9% confidence intervals as indicated in the text.

## RESULTS

**Purification of FomA.** Recombinant FomA (28, 52) was purified following the method that our group has developed for purification of LPS-free and lipoprotein-free neisserial porins (41, 46). *E. coli* FomA-containing inclusion bodies were solubilized by adding the zwitterionic detergent Zwittergent 3-14, and the protein purification was carried out in the presence of detergent. A three-step process was applied, consisting of ion exchange, size exclusion, and affinity chromatography. Following ion-exchange chromatography on DEAE resin, the majority of FomA was recovered in the column flowthrough, as detected by SDS-PAGE and Coomassie staining (not shown). The FomA-containing material was concentrated and applied to a Sephacryl 300 column, granting separation from protein contaminants of different molecular sizes, as shown in Fig. 1A. Fractions from the observed peaks were examined by SDS-PAGE and Coomassie staining; a band with a molecular size of approximately 40 kDa was identified in the major elution peak (Fig. 1B, arrow), along with minor contaminant bands of smaller molecular size. Enriched FomA-containing fractions were pooled and applied to a Matrex cellulose column for removal of contaminating endotoxin and lipopeptide (46). Purified FomA fractions, identified by SDS-PAGE and Coomassie staining (not shown), were concentrated, resuspended in 10% D-octyl-glucoside (DOG), and dialyzed extensively against PBS to allow formation of detergent-free protein micelles (termed proteosomes). The absence of *E. coli* LPS contamination was assessed by SDS-PAGE and silver staining, shown in Fig. 1C, and also confirmed by functional assays described below.

**FomA induces TLR2-dependent HEK cell activation.** Purified recombinant, LPS-free FomA (10  $\mu$ g/ml) was used to stimulate HEK cells overexpressing TLR2 (TLR2-HEK cells) or transfected with an empty vector, pcDNA-HEK cells, as a negative control. Induction of NF- $\kappa$ B-driven luciferase activity was measured after overnight incubation (15, 47) and expressed in arbitrary units  $\pm$  SD normalized to nonstimulated cells. In these cells, FomA in-

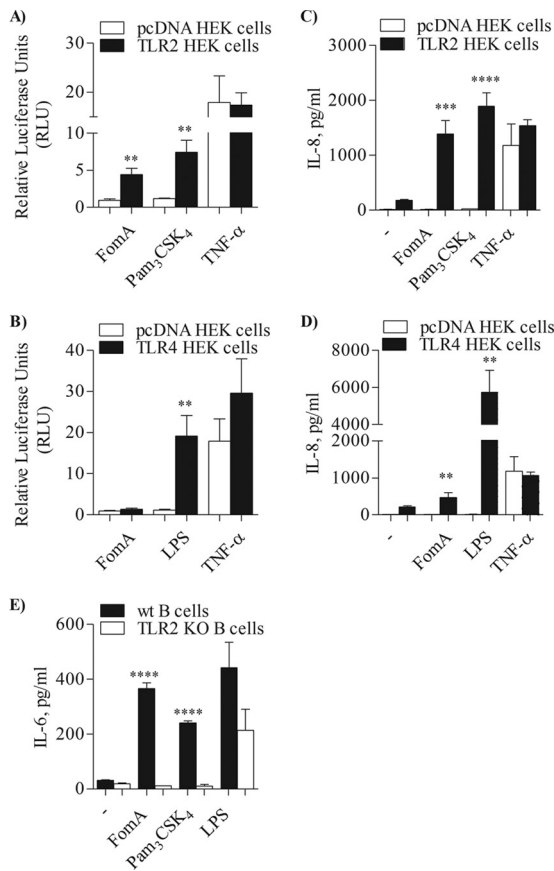


**FIG 1** FomA purification. (A) Representative chromatogram of FomA elution from gel filtration chromatography on Sephacryl S300 column. (B) SDS-PAGE and Coomassie staining of representative fractions from panel A. The FomA-containing fractions are indicated by the arrow. FomA was identified as a band of approximately 40 kDa in the major elution peak. (C) SDS-PAGE and silver staining of final FomA preparation. Lane 1, molecular weight standard. Lane 2, FomA, 2  $\mu$ g total. Lane 3, *E. coli* LPS, 2  $\mu$ g total.

duced high luciferase activity only when TLR2 was overexpressed, similar to the TLR2 agonist Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml) (Fig. 2A, black bars and white bars) (\*\*, *P* < 0.0023 by unpaired *t* test with Welch's correction; *n* = 12). As a non-TLR2-dependent control, cells were also incubated with recombinant human TNF- $\alpha$  (20 ng/ml), which induced high luciferase activity in both cell types. As another indicator of TLR2-dependent cell activation, secretion of IL-8 was measured by ELISA of cell supernatants and expressed as pg/ml  $\pm$  SD. Both FomA and Pam<sub>3</sub>CSK<sub>4</sub> induced high levels of IL-8 in TLR2-HEK cells but not in pcDNA-HEK cells (Fig. 2C, black bars and white bars, respectively) (\*\*\*, *P* = 0.0002; \*\*\*\*, *P* < 0.0001; *n* = 12), whereas both cell types were activated by TNF- $\alpha$ .

In contrast, while *E. coli* LPS (100 ng/ml) induced luciferase activity and IL-8 secretion in HEK cells overexpressing TLR4 (TLR4-HEK cells), these cells were not activated by FomA, consistent with the absence of endotoxin contamination observed by biochemical analysis of purified FomA. In fact, luciferase activity induced by LPS in TLR4-HEK cells was significantly higher than that induced in pcDNA cells (Fig. 2B, black bars and white bars) (\*\*, *P* = 0.0075; *n* = 9), as was IL-8 secretion (Fig. 2D, black bars and white bars) (\*\*, *P* = 0.0014; *n* = 9), while the effects of FomA were comparable in TLR4-HEK cells and pcDNA-HEK cells (Fig. 2B and D). In addition, TLR4-HEK cell activation induced by LPS was significantly higher than that induced by FomA (\*\*, *P* = 0.008). As expected, only TNF- $\alpha$  induced luciferase activity and IL-8 secretion in pcDNA-HEK cells (Fig. 2B and 2D, respectively, white bars).

**FomA induces TLR2-dependent mouse B cell activation *in vitro*.** Having demonstrated that purified recombinant FomA activates cells via TLR2 signaling and that it is free of endotoxin contamination, its ability to induce immune cell activation was examined using primary murine B cells. Splenic B cells were isolated from wild-type C57BL/6 mice (wt mice) and from TLR2



**FIG 2** TLR2-dependent cell activation by FomA. (A and B) TLR2-HEK cells and pcDNA-HEK cells (A) or TLR4-HEK cells and pcDNA-HEK cells (B) incubated with purified FomA (10  $\mu$ g/ml), Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml), *E. coli* LPS (100 ng/ml), and TNF- $\alpha$  (20 ng/ml) for 18 h. NF- $\kappa$ B-dependent luciferase activity was measured and is expressed in arbitrary units  $\pm$  SD normalized to nonstimulated cells. \*\*,  $P < 0.0023$  (for panel A) and  $P = 0.0075$  (for panel B). (C and D) IL-8 secretion measured by ELISA of supernatants of TLR2-HEK cells and pcDNA-HEK cells (C) and TLR4-HEK cells and pcDNA-HEK cells (D) incubated as described above. IL-8 is expressed as pg/ml  $\pm$  SD. \*\*\*,  $P = 0.0002$ ; \*\*\*\*,  $P < 0.0001$  (for panel C). \*\*,  $P = 0.0014$ ; \*\*\*,  $P = 0.008$  (for panel D). (E) IL-6 measured by ELISA of supernatant from purified splenic B cells from C57BL/6 mice (wt) and TLR2 KO mice stimulated with FomA, Pam<sub>3</sub>CSK<sub>4</sub>, and LPS as described above. IL-6 is expressed as pg/ml  $\pm$  SD. \*\*\*\*,  $P < 0.0001$ .

knockout mice (TLR2 KO mice) and incubated for 18 h with FomA (10  $\mu$ g/ml), Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml), and *E. coli* LPS (100 ng/ml). Induction of IL-6 secretion was measured by ELISA of cell supernatants, shown in Fig. 2E. IL-6 induced by FomA was comparable to that of Pam<sub>3</sub>CSK<sub>4</sub> in B cells from wt mice (Fig. 2E, black bars) but was significantly lower in B cells from TLR2 KO mice (Fig. 2E, white bars) (\*\*\*\*,  $P < 0.001$  by unpaired *t* test). As expected, IL-6 induced by LPS was comparable in B cells from wt mice and TLR2 KO mice (Fig. 2E).

Next, the ability of FomA to induce upregulation of surface markers indicative of B cell activation was examined by flow cytometry. Following incubation as described above, B cells were collected and stained with FITC-labeled mouse anti-CD86 and anti-MHC II antibodies. In all the histograms in Fig. 3, the isotype control is shown by the dotted line, the thin line represents cells incubated with medium alone, and the thick line represents stim-

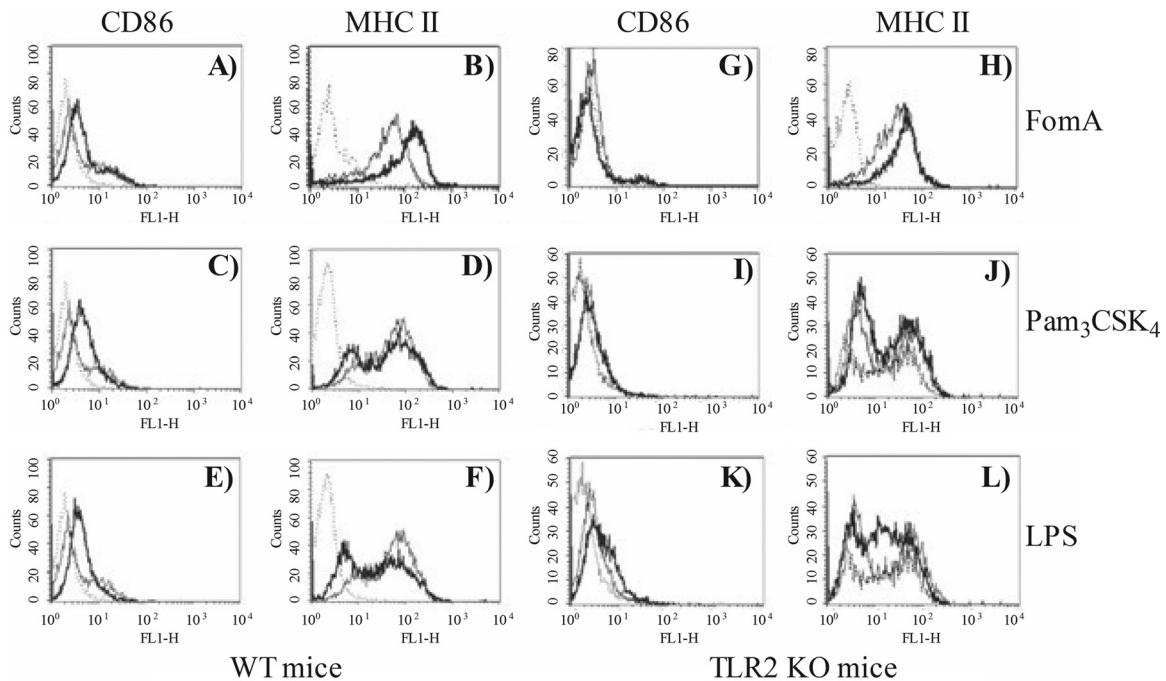
ulated cells. FomA, Pam<sub>3</sub>CSK<sub>4</sub>, and LPS upregulated surface expression of CD86 on the surface of B cells from wt mice (Fig. 3A, C, and E, histogram shift [thick line] versus baseline expression [thin line]). FomA also induced upregulation of MHC II surface expression (Fig. 3B); by contrast, in this experiment, the effect of Pam<sub>3</sub>CSK<sub>4</sub> and LPS appeared less substantial (Fig. 3D and F). In B cells from TLR2 KO mice, FomA and Pam<sub>3</sub>CSK<sub>4</sub> failed to induce CD86 and MHC II surface upregulation (Fig. 3G, H, J, and I). Stimulation with LPS was not affected by the lack of TLR2 expression (Fig. 3K and L).

**FomA induces TLR2-dependent antigen-specific antibody secretion *in vivo*.** To reevaluate the immune adjuvant activity of FomA and to rule out the potential contribution of LPS, mouse immunizations were performed using our LPS-free FomA and ovalbumin (OVA) (41). Wild-type C57BL/6 mice were immunized subcutaneously with OVA (10  $\mu$ g/mouse) alone and combined with LPS-free FomA (10  $\mu$ g/mouse) or with the non-TLR adjuvant, alum (200  $\mu$ g/mouse). The animals received three immunizations 2 weeks apart, and aliquots of preimmune sera and sera 2 weeks after each immunization (week 2, week 4, and week 6) were collected. Levels of serum anti-OVA specific antibodies were measured by ELISA and expressed in  $\mu$ g/ml  $\pm$  SD (41). Both anti-OVA IgG and IgM induced by immunization with OVA-FomA were significantly higher than those induced by OVA alone (Fig. 4A and B, dotted bars and white bars, respectively) (\*\*\*,  $P = 0.0005$  and  $0.0001$ , respectively, by unpaired *t* test). Similarly, both anti-OVA IgG and IgM induced by immunization with OVA-alum were significantly higher than those induced by OVA alone (Fig. 4C and D, dotted bars and white bars, respectively) (\*\*\*,  $P = 0.0001$ ; \*,  $P = 0.04$ ).

To examine whether the effect of FomA correlated with its observed TLR2-dependent *in vitro* activity, TLR2 KO mice were also immunized. As shown in Fig. 4E and F, low levels of specific anti-OVA IgG and IgM were detected in the sera of TLR2 KO mice immunized with OVA-FomA compared to sera of wt mice (dotted bars and white bars, respectively). As expected, anti-OVA IgG and IgM levels in TLR2 KO mice immunized with OVA-alum remained high, comparable to those induced in wt mice (Fig. 4G and H) (\*\*\*\*,  $P < 0.0001$ ; \*\*,  $P = 0.009$ ).

**Analysis of FomA-induced IgG subclasses and Th1/Th2 cytokine response.** Next, levels of IgG subclasses and Th1-type and/or Th2-type cytokines were analyzed in the sera of immunized mice. IgG subclasses were measured by ELISA and expressed as titers normalized to preimmune sera. In wt mice, immunization with OVA-FomA induced higher titers of anti-OVA-specific IgG1 than immunization with OVA alone (Fig. 5A, black bars and white bars, respectively) (\*\*\*,  $P = 0.0002$  by unpaired *t* test). FomA also induced increased levels of OVA-specific IgG2b and, although to a minor extent, IgG2c antibodies (IgG2c is the isotype present in C57BL/6 mice [44]) compared to OVA alone (Fig. 5A; \*,  $P = 0.03$ ). As expected, alum induced a prevalent IgG1 response (not shown) (41). Levels of OVA-specific IgG subclasses induced by immunization of TLR2 KO mice with OVA-FomA or OVA alone were comparable (Fig. 5B, black bars and white bars, respectively).

The cytokines IL-10 and IFN- $\gamma$  were chosen to evaluate the effect of FomA on the Th2 arm and the Th1 arm, respectively. A trend of increased levels of IL-10 was observed in the sera from wt mice immunized with OVA-FomA compared to sera of mice immunized with OVA alone (Fig. 5C, black bars and white bars,

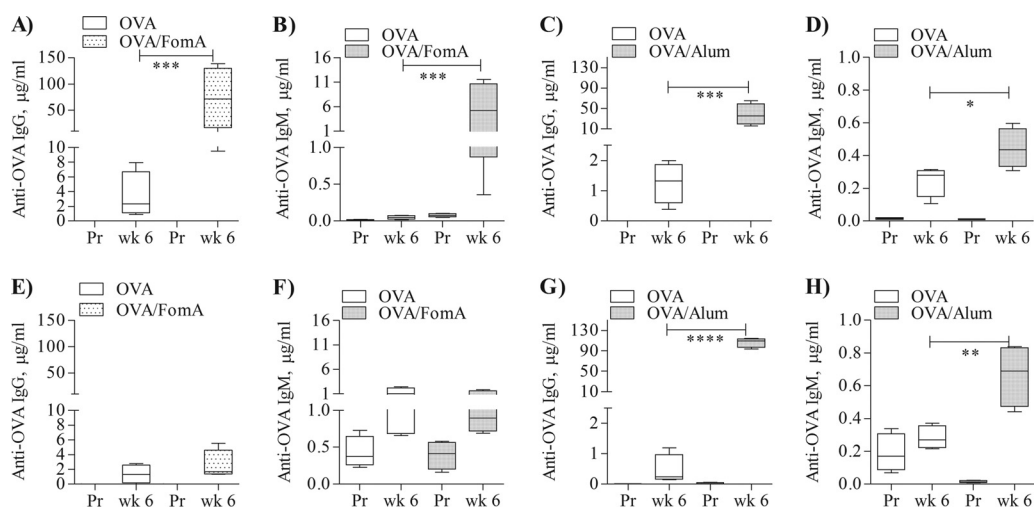


**FIG 3** Murine B cell activation *in vitro*. Purified splenic B cells from wt mice (A to F) and TLR2 KO mice (G to L) were incubated with 10  $\mu\text{g}/\text{ml}$  of FomA (A, B, G, and H), 100 ng/ml of Pam<sub>3</sub>CSK<sub>4</sub> (C, D, J, and I) or 100 ng/ml of *E. coli* LPS (E, F, K, and L) for 18 h. Surface upregulation of CD86 and MHC II was measured by flow cytometry. In all the histograms, the isotype control is indicated by the dotted line, cells incubated with medium alone are represented by the thin line, and stimulated cells are represented by the thick line.

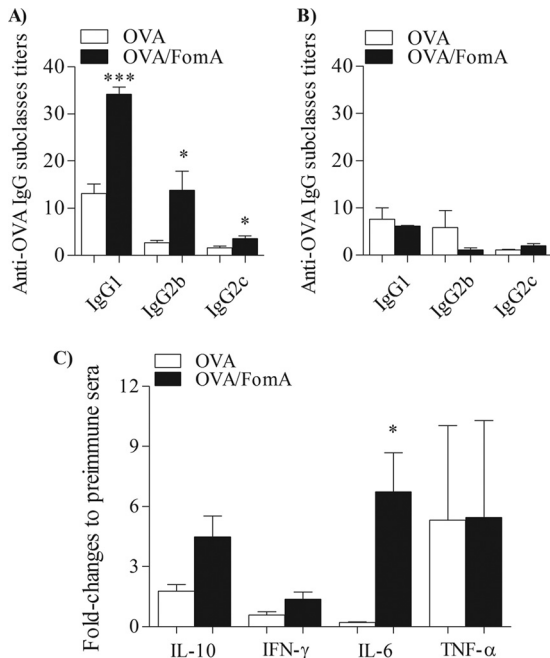
respectively) ( $P = 0.0506$ ). Although a slight increase in IFN- $\gamma$  secretion was measured in wt mice immunized with OVA-FomA compared to mice immunized with OVA alone, this was also non-statistically significant and of overall low magnitude (Fig. 5C). The cytokine IL-6, which promotes Th2 differentiation and simultaneously inhibits Th1 polarization (17), was significantly increased in the sera of OVA-FomA-immunized mice compared to those

immunized with OVA alone (Fig. 5C; \*,  $P = 0.015$ ). No difference in serum levels of TNF- $\alpha$  was observed among the two mouse groups (Fig. 5C). Results are expressed as fold change of titers  $\pm$  SD normalized to pooled preimmune sera. In TLR2 KO mice, immunization with OVA-FomA did not increase cytokine secretion compared to immunization with OVA alone (not shown).

**TLR2-dependent immunogenicity of FomA.** Since FomA is



**FIG 4** Enhancement of antigen-specific antibody production by FomA. (A to D) wt mice immunized with ovalbumin (OVA) (10  $\mu\text{g}$ ) alone and in combination with FomA (10  $\mu\text{g}/\text{ml}$ ) or alum (200  $\mu\text{g}$ ). Preimmune sera (Pr) and sera after the final immunization (week 6) were examined by ELISA for quantification of OVA-specific total IgG and IgM levels. (A and B) Anti-OVA IgG (A) and anti-OVA IgM (B), expressed as  $\mu\text{g}/\text{ml} \pm$  SD relative to mice immunized with OVA alone and OVA-FomA. \*\*\*,  $P = 0.0005$  and  $0.0001$  by unpaired *t* test. (C and D) Anti-OVA IgG (C) and anti-OVA IgM (D), expressed as  $\mu\text{g}/\text{ml} \pm$  SD relative to mice immunized with OVA alone and OVA-alum. \*\*\*,  $P = 0.0001$ ; \*,  $P = 0.04$ . (E to H) TLR2 KO mice were immunized as described above and OVA-specific IgG (E and G) and IgM (F and H) were quantified by ELISA. \*\*\*\*,  $P < 0.0001$ ; \*\*,  $P = 0.009$ .



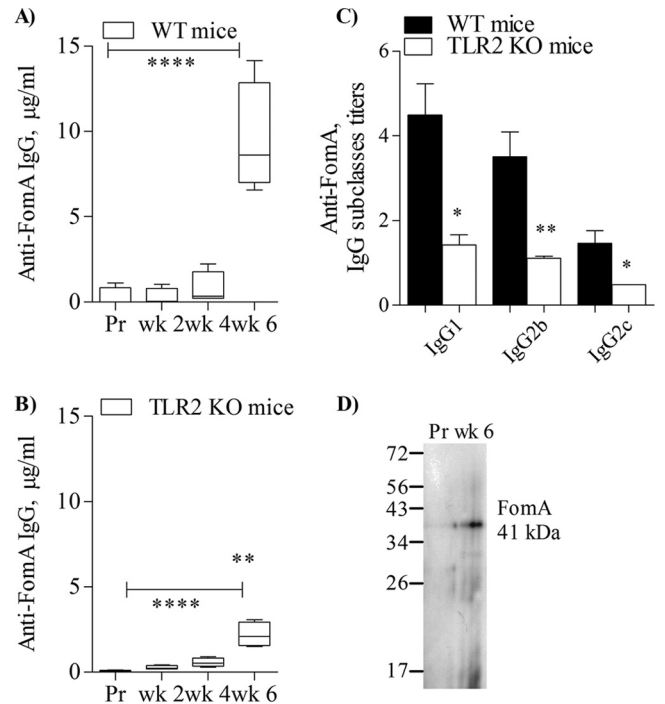
**FIG 5** Induction of antigen-specific IgG subclasses and cytokines by FomA. OVA-specific IgG subclasses measured in the sera of wt mice (A) and TLR2 KO mice (B) immunized with OVA alone or OVA-FomA by ELISA normalized to preimmune sera and expressed as mean titers  $\pm$  SD. \*,  $P = 0.03$ ; \*\*\*,  $P = 0.0002$ . Sera were used at a 1:100 dilution. (C) Cytokine levels in sera from wt mice immunized with OVA alone or OVA-FomA measured by ELISA. The values were normalized to pooled preimmune sera and expressed as fold change  $\pm$  SD. \*,  $P = 0.015$ .

known to be immunogenic (40, 50, 53), induction of specific anti-FomA antibodies was examined in the sera from mice immunized with FomA in the absence of other adjuvants. Sera were tested by ELISA using plates coated with FomA (2  $\mu$ g/ml), and IgG and IgM levels were expressed as  $\mu$ g/ml  $\pm$  SD.

As shown in Fig. 6A, anti-FomA total IgG production in wt mice increased in a dose-response fashion throughout the immunization and was significantly higher after the last boost (\*\*\*\*,  $P < 0.0001$  by one-way ANOVA with Dunnett's multiple comparison posttest and 99.9% confidence intervals). A similar statistically significant difference within the sera from TLR2 KO mice immunized with FomA was observed (Fig. 6B) (\*\*\*\*,  $P < 0.0001$ ), but when the amounts of anti-FomA IgG produced by wt mice and by TLR2 KO were compared, it was found that anti-FomA IgG levels in wt mice were significantly higher than those elicited in TLR2 KO mice (compare Fig. 6A and B; \*\*,  $P = 0.004$  by unpaired  $t$  test).

Analysis of anti-FomA IgG subclass titers revealed that IgG1 and IgG2b were the most prominent subclasses in wt mice and that both were significantly higher in these mice than in TLR2 KO mice (Fig. 6C, black bars and white bars, respectively) (\*,  $P = 0.02$ ; \*\*,  $P = 0.006$  by unpaired  $t$  test). A relatively small, but statistically significant increase of IgG2c was also detected in wt mice compared to TLR2 KO mice (Fig. 6C) (\*,  $P = 0.01$ ). IgG subclasses are expressed as titers normalized to preimmune sera  $\pm$  SD.

Anti-FomA sera also recognized FomA as a band of approximately 40 kDa in Western blotting experiments (Fig. 6D, representative wt mouse serum, week 6, 1:1,000 dilution).



**FIG 6** Immunogenicity of FomA. (A) Anti-FomA total IgG from wt mice immunized with FomA in the absence of additional adjuvants measured by ELISA. Preimmune sera (Pr) and sera after each boost (week 2, week 4, and week 6) are shown. Results are expressed in  $\mu$ g/ml  $\pm$  SD. \*\*\*\*,  $P < 0.0001$ . (B) Anti-FomA total IgG from TLR2 KO mice as in panel A. \*\*\*\*,  $P < 0.0001$ . \*\*,  $P = 0.004$  for comparison of week 6 results in panels A and B. (C) Anti-FomA IgG subclasses in the sera from wt mice and TLR2 KO mice. Sera were used at a 1:100 dilution. Results are expressed as titers normalized to preimmune sera  $\pm$  SD. \*,  $P = 0.02$ ; \*\*,  $P = 0.006$ ; \*,  $P = 0.01$  (left to right, respectively). (D) Western blot of FomA, detected as a band of  $\sim$ 40 kDa by sera from a representative FomA-immunized mouse (week 6, 1:1,000 dilution).

## DISCUSSION

Bacterial porins are well-established TLR2 agonists, in combination with the coreceptors TLR1 and TLR6, and their molecular mechanism of immune adjuvant activity has been explained via TLR2 signaling (8, 12, 47, 58). Another example of bacterial product with TLR2-dependent adjuvant activity is the *E. coli* heat-labile enterotoxin subunits [LT-IIa-B<sub>5</sub> and LT-IIb-B<sub>5</sub>] (16, 39). A strong *in vitro* immunobiological activity was reported for the FomA porin from *F. nucleatum* (62) by immune cell activation *in vitro* and adjuvant activity *in vivo*. Although a copious contamination (>12%) by *F. nucleatum* endotoxin (a classical TLR4 agonist [67]) was acknowledged in the native FomA preparation used in this study, FomA activated cells from both LPS-sensitive and LPS-nonresponder mice. It was thus concluded that its effect was not due to *F. nucleatum* LPS. However, following discovery of TLR signaling in response to specific bacterial components, such as LPS contamination poses a challenge for interpretation of the immune adjuvant activity of FomA. To date, no further *in vitro* or *in vivo* studies have been carried out to elucidate the mechanism of action of FomA.

More recent *in vitro* and *in vivo* evidence has indirectly suggested a role for TLR2 in the activity of FomA. This was indicated by TLR2-dependent IL-8 secretion, ROS production, and expression of antimicrobial peptides in response to FomA-containing

bacterial fractions in a variety of cell systems (cell lines, gingival epithelial cells, and primary murine macrophages) (27, 29, 33, 43, 51). None of these responses have been attributed to *F. nucleatum* LPS or peptidoglycan (a classical TLR2/TLR6 agonist [29, 38, 51]), while little is known about *Fusobacterium* lipoproteins, except their predicted existence (5, 32).

Thus, the immune stimulatory properties of FomA were examined using a recombinant, LPS-free FomA obtained using a method that warrants removal of LPS and lipoprotein contaminants (41, 46). Our results show that this FomA induced TLR2-dependent cell activation in a human nonhematopoietic cell model of TLR expression (HEK cells) and in purified splenic B cells from wild-type and TLR2 KO mice. In HEK cells, FomA induced IL-8 secretion and NF- $\kappa$ B-dependent luciferase activity only in cells in which TLR2 was overexpressed, whereas it did not activate HEK cells overexpressing TLR4. This observation indicates that FomA signals via TLR2 and confirms the absence of LPS contamination (*E. coli* LPS is a known TLR4 ligand [15]). FomA also induced IL-6 secretion and cell surface upregulation of the costimulatory molecule CD86 and of MHC II *in vitro* in B cells from wild-type mice but failed to activate B cells from TLR2 KO mice, similar to the TLR2 agonist Pam<sub>3</sub>CSK<sub>4</sub>. Our studies thus provide the first direct evidence of TLR2-dependent activity for FomA.

The adjuvanticity of FomA was also reevaluated *in vivo*, and FomA was shown to enhance antibody production to OVA when used in immunization of wt mice, similar to the effect of the adjuvant alum. However, in TLR2 KO mice, FomA, but not alum, failed to improve the specific response to OVA.

It is generally accepted that, in vaccinations, the adjuvant's nature contributes to modify local inflammatory responses and infection outcomes based on its effect on T-cell polarity. High levels of antibodies and isotype class switch toward IgG1 are considered features of the Th2-type immune response, along with anti-inflammatory cytokines IL-6, IL-4, and IL-10 (49). Such responses are classically induced by alum (11) and by TLR2 adjuvants (30). In contrast, production of IgG2a, IgG2b, IgG2c, and IgG3 (depending on the mouse strain [44]), as well as proinflammatory cytokines IFN- $\gamma$ , IL-2 and IL-12, indicates induction of Th1-type immune responses, favored by TLR4 and TLR9 adjuvants. Remarkably, in the past few years, increasing evidence has demonstrated that immune cell activation via TLR2 is involved in shaping both types of immune responses (22, 59). In wild-type mice, both alum and FomA induced OVA-specific IgG1 antibodies (important for complement-independent pathogen neutralization). However, FomA also induced increased titers of anti-OVA IgG2b (important for complement fixation and both complement- and Fc-mediated bacterial opsonophagocytosis). FomA also induced TLR2-dependent production of IL-10, in the serum from mice immunized with FomA/OVA, and of IL-6, which plays a dual role in controlling Th1/Th2 differentiation by promoting Th2 responses and inhibiting Th1 differentiation (17). Our results strongly indicated that FomA is a TLR2 agonist with a classical TLR2-dependent immune adjuvant activity.

The immunogenicity of FomA was also assessed. Previously, production of circulating and salivary anti-FomA IgG and IgA antibodies was demonstrated following intranasal immunization of mice with whole *E. coli* transfected to express FomA or with purified FomA plus cholera toxin (CT) (a strong mucosal non-TLR-dependent adjuvant) (26, 34, 40, 50). In our subcutaneous

immunization of mice with purified FomA in the absence of additional adjuvants, production of anti-FomA IgG antibodies was also found to be dependent on TLR2 expression and was detected only in wt mice, in agreement with the notion that FomA has TLR2-dependent activity. High levels of FomA-specific IgG1 and IgG2b were observed. The immune mouse sera also recognized linear epitopes of FomA by Western blotting, in addition to conformational epitopes by ELISA.

Enhancement of systemic responses is crucial for development of protective immunity to infections, but immunization with soluble antigens requires the addition of adjuvants to be effective. Our studies demonstrate that FomA is a novel potential TLR2 adjuvant. In addition, FomA is moderately immunogenic. Given the significantly high enhancement of antigen-specific immune responses induced by FomA *in vivo* and its observed moderate immunogenicity, FomA could represent a valuable self-adjuvanted element in a multicomponent vaccine against mixed infections caused by *F. nucleatum* and other oral pathogens.

## ACKNOWLEDGMENTS

This work was supported by the Basic Research Transitional Program Award from the Boston University School of Medicine, Section of Infectious Diseases.

We thank J. H. Kleinschmidt, University of Konstanz, Germany, for the FomA-expressing *E. coli* strain.

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