

Toll-Like Receptor 2-Dependent Activity of Native Major Outer Membrane Protein Proteosomes of *Chlamydia trachomatis*

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Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen and the etiologic agent of blinding trachoma. Intracellular signaling pathways leading to host cell inflammation and innate immunity to *Chlamydia* include those mediated by Toll-like receptors (TLRs) and nucleotide binding oligomerization domain 1 (Nod1) protein. In epithelial cells, TLR-dependent signaling contributes to local immune responses via induction of inflammatory mediators. There is evidence that TLR3, TLR4, and, particularly, TLR2 are critical for *Chlamydia*-mediated host cell activation and pathology. Despite the importance of TLR2, major chlamydial TLR2 antigens have not been identified so far. Numerous bacterial porins are known TLR2 agonists, i.e., porins from *Neisseriae*, *Shigella*, *Salmonella*, *Haemophilus influenzae*, and *Fusobacterium nucleatum*, which share structural and functional similarities with the chlamydial major outer membrane protein (MOMP), a strong antigen candidate for a potential vaccine against *C. trachomatis*. We describe the ability of purified, detergent-free MOMP to signal via TLR2 *in vitro* in TLR-overexpressing cells and TLR2-competent human reproductive tract epithelial cell lines. Using MOMP formed in pure protein micelles (proteosomes), we show the induction of TLR2-dependent interleukin-8 (IL-8) and IL-6 secretion *in vitro*, the involvement of TLR1 as a TLR2 coreceptor, and the activation of both NF- κ B and mitogen-activated protein (MAP) kinase intracellular pathways. Interestingly, MOMP proteosomes induce cytokine secretion in endocervical epithelial cells (End/E6E7) but not in urethral epithelial cells (THUECs). A detailed understanding of the TLR2-dependent molecular mechanisms that characterize the effect of MOMP proteosomes on host cells may provide new insights for its successful development as an immunotherapeutic target against *Chlamydia*.

The role of Toll-like receptors (TLRs; cell surface and intracellular receptors that recognize microbial products called pathogen-associated molecular patterns [PAMPs]) (1) in modulation of host innate and adaptive immunity by bacterial products is well established. TLR engagement and signaling induce activation of intracellular signaling pathways such as NF- κ B and mitogen-activated protein (MAP) kinases that regulate secretion of inflammatory mediators by both nonhematopoietic cells and immune cells, as well as expression of costimulatory ligands and MHC molecules, ultimately enhancing the host humoral and cellular immune responses (1, 2). Among the known bacterial components with TLR agonist function, lipopolysaccharide (LPS) activates TLR4 in complex with MD2 (3), flagellin activates TLR5 (4), and CpG DNA and double-stranded RNA (dsRNA) engage TLR9 and TLR3, respectively (5, 6). A much broader repertoire of ligands characterizes TLR2, due to its heterodimerization with TLR1 or TLR6. Examples of bacterial TLR2 agonists are lipopeptides (7), peptidoglycans (8) and porins (i.e., from *Neisseriae*) (9, 10), *Shigella* (11), *Salmonella* (12), *Haemophilus influenzae* (13), and *Fusobacterium nucleatum* (14).

Chlamydia species are associated with a number of diseases. *C. trachomatis* is the most common sexually transmitted bacterial pathogen and up to 4 million to 5 million new genital *C. trachomatis* infections are reported annually in the United States (15). While genital infections can remain asymptomatic in women, long-term sequelae, such as infertility and ectopic pregnancy, can develop (16). *C. trachomatis* is also the etiologic agent of blinding trachoma, which is one of the leading causes of blindness in the world (17, 18). *C. pneumoniae* is a cause of community-acquired pneumonia and pharyngitis (approximately 6 to 10% of community-acquired pneumonia cases) (19) and may play a role in

chronic inflammatory conditions, such as asthma, reactive arthritis, and atherosclerosis (20).

Chlamydia infection activates several innate immune pathways, leading to host cell responses, including TLR signaling (21) and the activation of the nucleotide binding oligomerization domain 1 (Nod1) protein (22), an intracellular pattern recognition receptor (23). For example, evidence of TLR3 activation by *Chlamydia* has been reported in murine reproductive tract epithelial cells (24), and there is strong evidence that TLR2 is critical for *Chlamydia*-mediated host cell activation and pathology (25, 26). However, only a few chlamydial components have been implicated in TLR-dependent cell activation, for example, the lipoprotein macrophage infectivity potentiator Mip, which signals via TLR2/TLR1/TLR6 and CD14 (27), and LPS, which signals via TLR4 (although with weaker activity than *Escherichia coli* LPS) (28, 29). A number of studies have also reported signaling via both TLR4 and TLR2 for chlamydial heat shock protein 60 (hsp60) *in vitro* and *in vivo* (30–32). Although the importance of TLR2 for the induction of host immune responses to *Chlamydia* infection and for *Chlamydia* virulence is recognized (21, 33–35), major chlamydial TLR2 antigens have not been identified so far. For

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example, expression of peptidoglycan (a TLR2 agonist) is considered to be minimal or even undetectable in *Chlamydia* (36, 37).

Porins, the most abundant outer membrane proteins of Gram-negative organisms, are established TLR2 ligands. Bacterial porins, including the chlamydial major outer membrane porin protein (MOMP), constitute a high percentage of the total outer membrane protein content (over 60%) (38) and share structural and functional similarities among organisms. *C. trachomatis* MOMP is surface exposed, has a molecular mass of ~40 kDa in monomeric form, and is found in homotrimeric form in the bacterial outer membrane (39, 40). It is immunogenic (41) and can induce protection in the mouse and monkey models (42, 43). Since MOMP is considered a strong antigen candidate for a *Chlamydia* vaccine, a detailed understanding of the TLR-dependent molecular mechanisms that characterize its activity on host cells will provide new insights for its successful development as an immunotherapeutic target against *Chlamydia*. However, as for all purified bacterial integral membrane proteins, the addition of detergents is necessary to maintain native conformation and solubility in aqueous solutions. Due to the cytotoxic nature of detergents, it has proved difficult to establish the effects of native purified MOMP *in vitro* so far. To overcome this obstacle, in the present study, we have examined the ability of purified *C. trachomatis* MOMP formed in pure protein micelles (proteosomes) to induce inflammatory responses *in vitro* in a variety of cell models relevant for *C. trachomatis* infection. Using both a TLR2 overexpression cell model (HEK cells) and human reproductive tract epithelial cell lines, we have established that MOMP proteosomes induce cell activation via TLR2 signaling and that TLR1 is the necessary TLR2 coreceptor for its activity, while TLR4 does not play a major role in the activity of MOMP proteosomes. Furthermore, we show that interleukin-8 (IL-8) secretion is induced by MOMP proteosomes via multiple intracellular signaling pathways, namely, NF- κ B nuclear translocation and the activation of MAP kinases. Interestingly, our results show that purified MOMP proteosomes induce secretion of IL-8 and IL-6 in the endocervical epithelial cell line End/E6E7 *in vitro*, but these proinflammatory mediators are not induced in human urethral epithelial cells.

MATERIALS AND METHODS

Preparation of native *Chlamydia muridarum* mouse pneumonitis (MoPn) MOMP proteosomes. *Chlamydia muridarum* (strain Nigg II; previously called *Chlamydia trachomatis* mouse pneumonitis [ATCC]) was grown in McCoy cells (44). The extraction and purification of native MOMP were performed as described previously (40). In brief, after extraction of MOMP from bacterial lysates using 2% Anzergent 3-14 (*n*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) (Z3-14), the protein suspension was subject to column chromatography on hydroxyapatite resin (Bio-Gel HTP Gel) in the presence of 0.1% Z3-14. Purified MOMP, identified by electrophoresis on 10% Tricine-SDS-PAGE and Western blotting using the monoclonal antibody MoPn40 to MOMP (40), was concentrated and 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% NaN₃ for the formation of detergent-free protein micelles (proteosomes) (45). The protein concentration was determined using the bicinchoninic acid (BCA) assay.

Cell culture and growth conditions. Stably transfected HEK cells overexpressing TLR2, TLR4, TLR2/TLR1, TLR2/TLR6, or an empty vector (pcDNA) (3) were cultured at 37°C in a 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 μ g/ml ciprofloxacin. HeLa cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine,

100 U/ml penicillin, and 100 μ g/ml streptomycin. The endocervical cell line End/E6E7 (46) was cultured in keratinocyte serum-free medium (KFSM) (GIBCO-Invitrogen) containing 50 μ g/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor, 0.4 mM CaCl₂, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Transduced human urethral epithelial cells (THUECs) (47) were grown in prostate epithelial growth medium (PrEGM) (Cambrex) supplemented with 5% FBS.

Cell stimulation. A total of 10⁵ cells/ml of HEK cells, End/E6E7 cells, HeLa cells, or THUECs were plated in 100 μ l in 96-well plates and incubated with purified MOMP proteosomes (100, 10, or 1 μ g/ml), Pam₃CSK₄ or Pam₂CSK₄ (0.1 μ g/ml) (InvivoGen), lipoprotein-free *E. coli* LPS (0.1 μ g/ml) (Sigma), or recombinant human tumor necrosis factor alpha (TNF- α) (0.02 μ g/ml) (eBioscience) in triplicate wells for 3 h, 6 h, or 24 h. For MAP kinase signaling pathway inhibition, cells were treated with 25 μ M (in 10 mM dimethyl sulfoxide [DMSO]) U0126 (MEK1/2 inhibitor, upstream kinase for ERK1/2 phosphorylation), SB203580 (inhibitor of p38 phosphorylation), or SP600125 (inhibitor of Jun N-terminal protein kinase [JNK] phosphorylation) (Sigma) or 25 μ g/ml of an NF- κ B inhibitory ligand (Millipore) for 1 h prior to cell stimulation.

Cytokine ELISA. IL-8 and IL-6 secretion was measured by an enzyme-linked immunosorbent assay (ELISA) of supernatants from cell cultures stimulated as described above using OptEIA kits (BD Biosciences) per the manufacturer's protocol.

NF- κ B luciferase reporter assay. HEK cells were transiently transfected with an NF- κ B luciferase reporter vector as described previously (3). 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. Results are expressed as relative luciferase units \pm standard errors.

Statistical analysis. Statistical analyses were calculated using Graph-Pad PRISM software. *P* values were calculated using an unpaired *t* test with Welch's correction, a one-sample *t* test, and a one-way analysis of variance (ANOVA) with a Bonferroni posttest, as described in Results.

RESULTS

Analysis of native purified *C. muridarum* MoPn MOMP and MOMP proteosomes. Purified MOMP was obtained by chromatographic separation in the presence of detergent as described previously (40). Following purification, to maintain the protein in native trimeric conformation while removing the detergent to allow its use in cell-based *in vitro* assays, MOMP was concentrated, resuspended in 10% D-octyl-glucoside (DOG) and dialyzed extensively against PBS to allow the formation of detergent-free and lipid-free protein micelles (termed proteosomes). To assess whether inclusion of MOMP into proteosomes affected the trimeric state of the purified protein, aliquots of native purified (MoPn) MOMP and MOMP proteosomes were compared by electrophoresis on a 10% Tricine-SDS-PAGE. In Fig. 1A, purified *Chlamydia* MoPn MOMP (containing detergent) was heat denatured in the presence of 25 mM dithiothreitol (DTT) prior to electrophoresis and a band of approximately 39 kDa, corresponding to the monomeric form (40, 48), was observed (Fig. 1A, lane 2). When the purified MOMP was not subject to heat denaturation, a major band with an apparent molecular mass of 66 kDa, typically indicative of MOMP trimeric forms (40, 48), was observed both in the presence and in the absence of DTT (Fig. 1A, lanes 3 and 4, respectively). Furthermore, additional bands of high molecular weights were detected in the absence of DTT (Fig. 1A, lane 4).

Next, detergent-free MOMP proteosomes were examined by

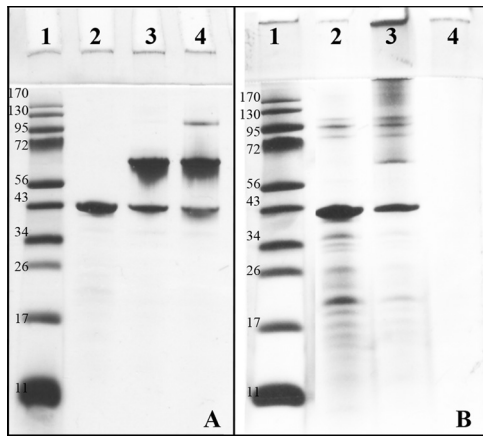


FIG 1 Comparison of purified *Chlamydia* MoPn MOMP and MOMP proteosomes. (A) Ten percent Tricine-SDS-PAGE of purified *Chlamydia* MoPn MOMP. Lane 1, molecular weight standard; lane 2, heat-denatured, purified MOMP containing 25 mM DTT; lane 3, not heat-denatured, purified MOMP with 25 mM DTT; and lane 4, not heat-denatured, purified MOMP in the absence of DTT. (B) Ten percent Tricine-SDS-PAGE of MOMP proteosomes. Lane 1, molecular weight standard; lane 2, heat-denatured MOMP proteosomes containing 25 mM DTT; lane 3, not heat-denatured MOMP proteosomes containing 25 mM DTT; and lane 4, not heat-denatured MOMP proteosomes in the absence of DTT.

10% Tricine-SDS-PAGE. In Fig. 1B, when MOMP proteosomes were heat denatured in the presence of 25 mM DTT, the monomer band (39 kDa) was mostly detected (Fig. 1B, lane 2), as were some minor bands at low molecular weights (possibly degradation products). When MOMP proteosomes were not heat denatured prior to electrophoresis in the presence of 25 mM DTT, the monomeric form was observed along with additional bands of high molecular weights, i.e., the 66-kDa band indicative of trimers, and possibly oligomeric forms (Fig. 1B, lane 3). Lastly, when the MOMP proteosome preparation was not heat denatured and no DTT was added, the sample failed to enter the gel due to the lack of denaturation (Fig. 1B, lane 4).

MOMP proteosomes induce IL-8 secretion *in vitro* via TLR2.

MOMP proteosomes were used to stimulate HEK cells overexpressing TLR2 (TLR2-HEK cells) or cells transfected with an empty vector as a negative control (pcDNA-HEK cells). After 24 h, IL-8 secretion was assessed by ELISA of culture supernatants and is expressed in pg/ml. While MOMP proteosomes (10, 1, and 0.1 μ g/ml) failed to stimulate pcDNA-HEK cells (Fig. 2A, white bars), a dose-dependent increase of IL-8 production was induced in TLR2-HEK cells (Fig. 2A, black bars) (**, $P = 0.001$, and ****, $P < 0.0001$, by unpaired t test with Welch's correction). As expected, the TLR2 agonist Pam₃CSK₄ (0.1 μ g/ml) induced high levels of IL-8 secretion in TLR2-HEK cells (Fig. 2A, black bars), which were higher than those induced by MOMP proteosomes (**, $P = 0.006$, and ****, $P < 0.0001$). In both cell types, TNF- α (0.02 μ g/ml) induced equivalent IL-8 production in a TLR-independent manner (Fig. 2A, white bars and black bars). To assess whether TLR4 contributed to the effect of MOMP proteosomes, HEK cells overexpressing TLR4 were also used. In these cells, *E. coli* LPS (0.1 μ g/ml) induced significantly higher levels of IL-8 secretion than MOMP proteosomes and Pam₃CSK₄ (Fig. 2B, black bars) (***, $P = 0.0008$, and **, $P = 0.0012$, respectively), while the effect of MOMP proteosomes and Pam₃CSK₄ was comparable to that of incubation with medium alone. An apparently high baseline of

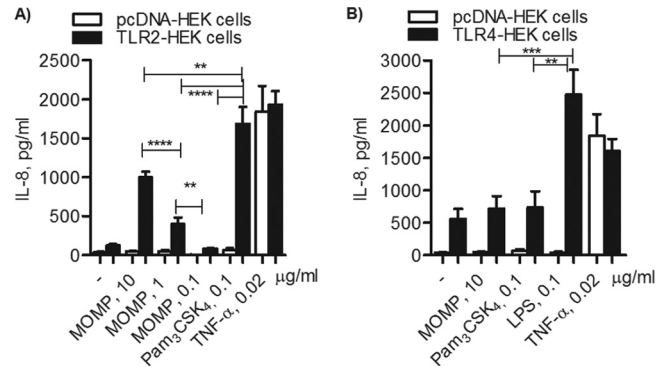


FIG 2 TLR2-dependent IL-8 induction by MOMP proteosomes. (A) TLR2-HEK cells (black bars) and pcDNA-HEK cells (white bars) incubated with MOMP proteosomes (10, 1, and 0.1 μ g/ml), Pam₃CSK₄ (0.1 μ g/ml), *E. coli* LPS (0.1 μ g/ml), or TNF- α (0.02 μ g/ml) for 18 h. IL-8 secretion was measured by ELISA of cell culture supernatants and expressed as pg/ml \pm standard error (**, $P = 0.001$, **, $P = 0.006$, and ****, $P < 0.0001$, by unpaired t test with Welch's correction; $n > 20$). (B) TLR4-HEK cells (black bars) and pcDNA-HEK cells (white bars) were incubated as described above (***, $P = 0.0008$, and **, $P = 0.0012$; $n > 10$).

IL-8 production was observed in TLR4-HEK cells, likely due to the presence of soluble MD-2 provided by the FBS-containing cell culture medium, leading to nonspecific stimulation.

MOMP proteosomes induce luciferase activity *in vitro* via TLR2. As an additional indicator of cell activation, induction of NF- κ B-driven luciferase activity was also measured in HEK cells following stimulation with MOMP proteosomes. Luciferase activity is expressed as arbitrary relative luciferase units (RLU) normalized to nonstimulated cells \pm standard errors. In TLR2-HEK cells, both MOMP proteosomes (10 μ g/ml) and Pam₃CSK₄ (0.1 μ g/ml) induced comparable levels of luciferase activity (Fig. 3A, black bars) ($P > 0.05$), which was predictably significantly higher than that induced by *E. coli* LPS (0.1 μ g/ml) (**, $P = 0.002$). None of the stimuli used induced luciferase activity in pcDNA-HEK cells, except for TNF- α (0.02 μ g/ml) (Fig. 3A and B, white bars), via a TLR-independent mechanism. Similar to IL-8, significantly high luciferase activity was induced in TLR4-HEK cells by *E. coli* LPS

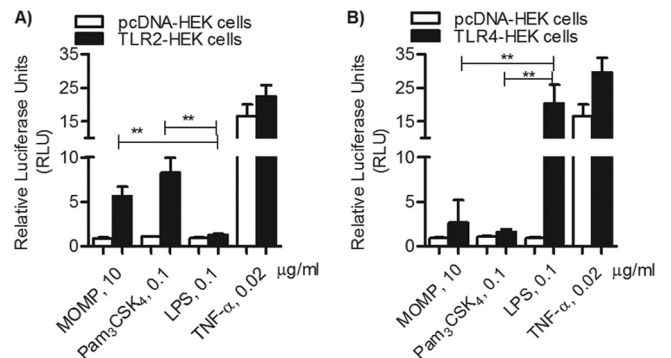


FIG 3 TLR2-dependent NF- κ B luciferase activity. (A) TLR2-HEK cells (black bars) and pcDNA-HEK cells (white bars) incubated with MOMP proteosomes (10 μ g/ml), Pam₃CSK₄ (0.1 μ g/ml), *E. coli* LPS (0.1 μ g/ml), or TNF- α (0.02 μ g/ml) for 18 h. Luciferase activity was measured and is expressed in relative luciferase units (RLU) \pm standard error normalized to nonstimulated cells (**, $P = 0.002$, by unpaired t test with Welch's correction; $n = 6$). (B) TLR4-HEK cells (black bars) and pcDNA-HEK cells (white bars) incubated as described above (**, $P = 0.006$ and 0.003; $n = 9$).

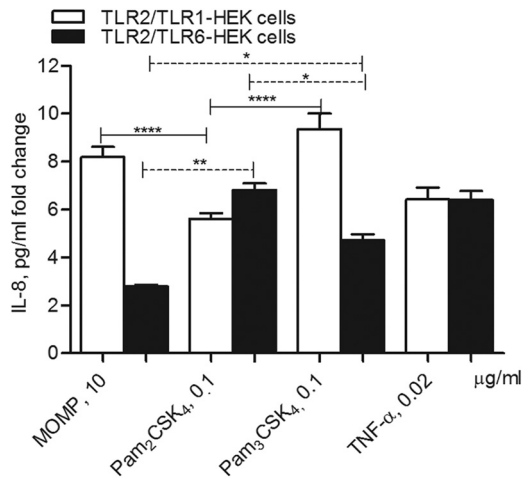


FIG 4 MOMP proteosome signal via TLR2/TLR1. HEK cells overexpressing TLR2/TLR1 (white bars) or TLR2/TLR6 (black bars) incubated with MOMP proteosomes (10 µg/ml), Pam₂CSK₄ (0.1 µg/ml), Pam₃CSK₄ (0.1 µg/ml), and TNF-α (0.02 µg/ml) for 18 h. IL-8 secretion was measured by ELISA of cell culture supernatants and expressed as a pg/ml fold change normalized to cells incubated with medium alone ± standard errors. For TLR2/TLR1-HEK cells, ****, $P < 0.0001$, by unpaired t test with Welch's correction; $n = 19$. For TLR2/TLR6-HEK cells, **, $P = 0.005$, and *, $P = 0.01$.

(0.1 µg/ml) compared to MOMP proteosomes and Pam₃CSK₄ (Fig. 3B, black bars) (**, $P = 0.006$ and 0.003).

MOMP proteosomes signal via TLR2/TLR1. TLR2 heterodimerization with TLR1 or TLR6 is required for the specific recognition of different ligands and for the induction of cell activation. To identify the TLR2 coreceptor necessary for MOMP activity *in vitro*, IL-8 induction was measured in HEK cells overexpressing the TLR2/TLR1 or TLR2/TLR6 receptor pair and expressed as a pg/ml fold change normalized to cells incubated with medium alone. To assess stimulation via TLR2/TLR1, Pam₃CSK₄ was used as a positive control, while stimulation via TLR2/TLR6 was determined using Pam₂CSK₄ as a positive control. In Fig. 4, TLR2/TLR1-HEK cells are indicated by the white bars. In these cells, MOMP proteosomes and Pam₃CSK₄ induced comparable IL-8 levels ($P > 0.05$ by unpaired t test with Welch's correction) and both were significantly higher than those with Pam₂CSK₄ (****, $P < 0.0001$), consistent with TLR2/TLR1-mediated cell stimulation.

In HEK cells overexpressing TLR2 and TLR6 (Fig. 4, black bars), Pam₂CSK₄ induced significantly higher IL-8 levels than MOMP proteosomes (**, $P = 0.005$), suggesting cell activation via different receptor complexes. In these cells, as expected, Pam₂CSK₄ also induced higher IL-8 levels than Pam₃CSK₄ (*, $P = 0.01$). It needs to be noted that HEK cells express low endogenous levels of TLR1 and TLR6 (9), which likely accounts for the baseline level of IL-8 secretion that is consistently observed when TLR2/TLR1-HEK cells or TLR2/TLR6-HEK cells are cross-stimulated with the reciprocal agonists as well as MOMP. For example, both MOMP proteosomes and Pam₃CSK₄ induce some IL-8 in TLR2/TLR6-HEK cells, with Pam₃CSK₄ being more potent than MOMP proteosomes (*, $P = 0.01$), similar to that observed in TLR2/TLR1-HEK cells.

MOMP proteosome-dependent activation of intracellular signaling pathways. IL-8 expression can be induced by the acti-

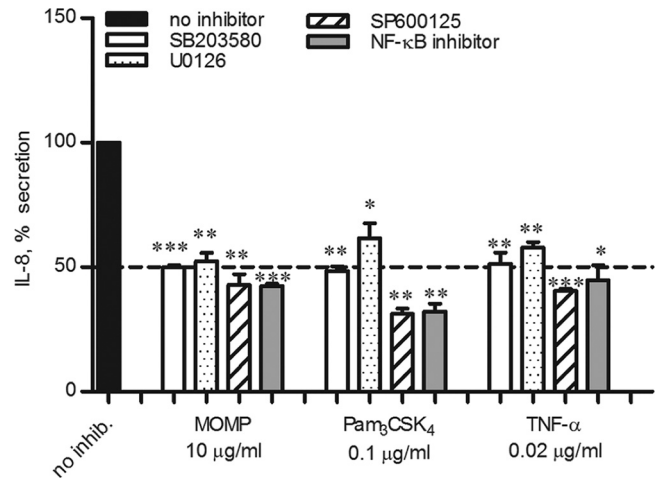


FIG 5 Intracellular cell signaling pathway inhibition. TLR2-HEK cells were incubated for 1 h with 25 µM p38 inhibitor SB203580 (white bars), ERK1/2 inhibitor U0126 (dotted bars), or JNK inhibitor SP600125 (striped bars) or 25 µg/ml of an NF-κB inhibitory ligand (gray bars) prior to stimulation with MOMP proteosomes (10 µg/ml), Pam₃CSK₄ (0.1 µg/ml), or TNF-α (0.02 µg/ml) for 24 h. Cells stimulated in the absence of inhibitors are indicated by the black bar. IL-8 secretion was measured by ELISA of cell supernatants, normalized to that induced in the absence of inhibitors and expressed as percent inhibition ± standard error. MOMP: ***, $P < 0.0003$, and **, $P < 0.006$; Pam₃CSK₄: **, $P < 0.002$, and *, $P = 0.02$; and TNF-α: ***, $P = 0.0001$, **, $P < 0.008$, and *, $P = 0.01$ (by a one-sample t test; $n = 3$).

vation of the NF-κB pathway and the AP-1 pathway (via ERK1/ERK2, JNK, and p38 MAP kinases) individually or in combination, depending on the cell type and the stimulus (49). To examine the contribution of each pathway to the effect of MOMP proteosomes *in vitro*, specific inhibitors were used in TLR2-HEK cells. Cells were incubated with 25 µM SB203580 (p38 inhibitor), U0126 (ERK1/2 inhibitor), or SP600125 (JNK inhibitor) or with 25 µg/ml of an NF-κB inhibitory ligand for 1 h prior to stimulation with MOMP proteosomes (10 µg/ml), Pam₃CSK₄ (0.1 µg/ml), and TNF-α (0.02 µg/ml) for 24 h. Secretion of IL-8 was measured by ELISA, normalized to that induced in the absence of inhibitors and expressed as percentage ± standard error. When stimulation with MOMP proteosomes was carried out in the presence of each inhibitor (Fig. 5, SB203580, white bars; U0126, dotted bars; SP600125, striped bars; and NF-κB inhibitory ligand, gray bars), IL-8 secretion was reduced by approximately 50% compared to IL-8 induced in the absence of inhibitors (Fig. 5, black bar) (***, $P < 0.0003$, and **, $P < 0.006$, by a one-sample t test). Similarly, IL-8 induction by Pam₃CSK₄ was reduced by approximately 50% or more in the presence of SB203580, SP600125, and an NF-κB inhibitory ligand, while it was slightly less inhibited by U0126 (**, $P < 0.002$, and *, $P = 0.02$), consistent with previous results (50). Inhibition of TNF-α-induced IL-8 followed a similar trend, with a reduction of approximately 50% compared to TNF-α stimulation in the absence of inhibitors (***, $P = 0.0001$; **, $P < 0.008$; and *, $P = 0.01$). Thus, the ability of MOMP proteosomes to induce IL-8 secretion via TLR2 is mediated by the activation of multiple intracellular signaling pathways.

TLR2-dependent induction of inflammatory mediators by MOMP proteosomes in human reproductive tract epithelial cells. Evidence of TLR2-dependent signaling in the induction of inflammation in the female mouse model of genital tract *Chla-*

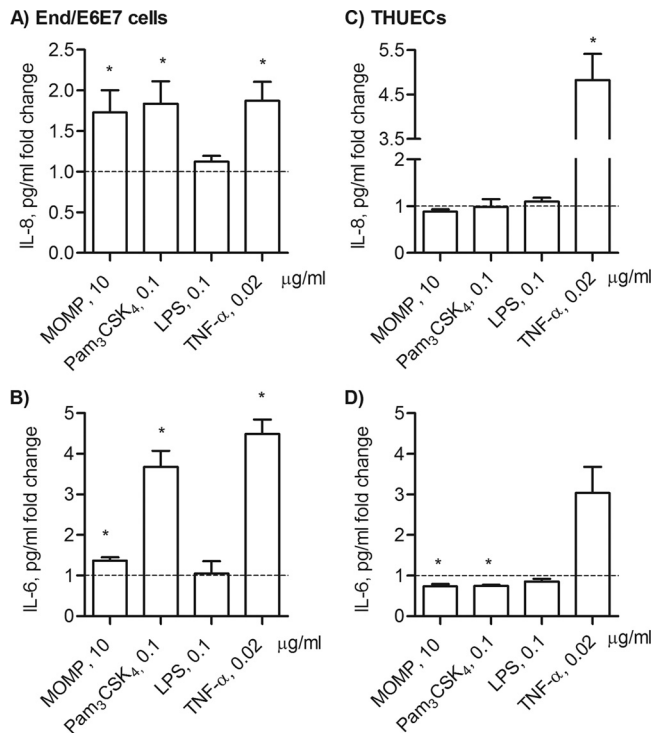


FIG 6 Inflammatory responses of human reproductive tract epithelial cells induced by MOMP proteosomes. IL-8 secretion (A) and IL-6 secretion (B) by End/E6E7 cells incubated with MOMP proteosomes (10 µg/ml), Pam₃CSK₄ (0.1 µg/ml), *E. coli* LPS (0.1 µg/ml), or TNF-α (0.02 µg/ml) for 24 h. Cytokines were measured by ELISA of cell supernatants and expressed as a pg/ml fold change relative to cells incubated with medium alone ± standard error (*, $P < 0.05$, by a one-sample *t* test; $n = 9$). IL-8 (C) and IL-6 (D) induced in THUECs incubated as described above (*, $P < 0.05$; $n = 3$).

mydia infection has been shown, and a role of TLR2 in site-specific mucosal inflammation has been hypothesized (35). To evaluate the effect of MOMP proteosomes in a naturally TLR2-competent cell model representative of the human female reproductive tract, the endocervical cell line End/E6E7 was used and secretion of inflammatory mediators IL-8 and IL-6 was examined. Due to high baseline cytokine secretion in growth factor-containing KSFM culture medium, IL-8 and IL-6 are expressed as a pg/ml fold change relative to cells incubated with medium alone. End/E6E7 cells, which lack expression of TLR4 and MD-2 (51), did not secrete IL-8 or IL-6 above the baseline level in response to stimulation with *E. coli* LPS (0.1 µg/ml) for 24 h (Fig. 6A and B). MOMP proteosomes and Pam₃CSK₄ induced a similar fold increase in IL-8 secretion (Fig. 6A) (*, $P < 0.05$, by a one-sample *t* test). In these cells, MOMP proteosomes also induced a statistically significant increase in IL-6 secretion, although the effect of Pam₃CSK₄ was more pronounced (Fig. 6B). As expected, TNF-α induced TLR2-independent IL-8 and IL-6 secretion (Fig. 6A and B).

The effect of MOMP proteosomes on HeLa cells, previously used as a cell model to study *Chlamydia* infection (52, 53), was also examined. However, since HeLa cells lack TLR1 expression and are nonresponsive to TLR2/TLR1 ligands (54, 55), no induction of IL-8 or IL-6 by MOMP proteosomes or by Pam₃CSK₄ was detected (not shown). In contrast to End/E6E7 cells, HeLa cells expressed TLR4 and were stimulated by *E. coli* LPS as well as by TNF-α in a TLR-independent manner (not shown). The re-

sponses of male urethral epithelial cells, THUECs (47, 52), were assessed. Similar to HeLa cells, no IL-8 secretion was induced in THUECs by MOMP proteosomes, Pam₃CSK₄, or even *E. coli* LPS incubation for 24 h (Fig. 6C). Furthermore, a below-baseline trend of IL-6 production was observed following incubation with MOMP proteosomes or Pam₃CSK₄ while TNF-α successfully induced IL-8 and IL-6 secretion (Fig. 6D). To determine whether inflammatory responses were elicited in End/E6E7 and THUECs by MOMP proteosomes with different kinetics, time-dependent IL-8 and IL-6 secretion was examined. In End/E6E7 cells, Pam₃CSK₄ and TNF-α induced high IL-8 secretion as early as 3 h and this remained sustained up to 24 h, while MOMP proteosomes only induced elevated IL-8 secretion at 24 h (Fig. 7A) (*, $P < 0.05$, by one-way ANOVA with a Bonferroni posttest). Stimulation of End/E6E7 cells with Pam₃CSK₄ for 3 h, 6 h, and 24 h showed a trend of increased IL-6 production over time (Fig. 7B), while an opposite trend was observed for TNF-α, where IL-6 secretion appeared reduced at 24 h compared to 3 h and 6 h (Fig. 7B). Similar to Pam₃CSK₄, MOMP proteosomes induced an apparent 2-fold increase in IL-6 secretion at 3 h and 6 h, but this remained unchanged (or possibly even slightly decreased) at 24 h (Fig. 7B). Conversely, in THUECs, sustained IL-8 and IL-6 secretion over time was only induced by TNF-α stimulation (Fig. 7C and D).

DISCUSSION

TLRs and other pattern recognition receptors (PRRs) recognize microbial products and modulate acute inflammatory responses, host innate and adaptive immune responses, and site-specific defense mechanisms. In the past decade, much progress has been made in defining how bacterial outer membrane components induce such responses via TLR signaling. For example, bacterial porins are well-established TLR2 agonists in combination with the coreceptors TLR1 and TLR6. The effect of numerous bacterial porins has been examined *in vitro* and *in vivo* (9–11, 13, 14), thus providing relevant information on how these products may influence initial mucosal host responses in specific cell niches by regulating TLR-dependent cell signaling.

A number of pattern recognition receptors, including TLRs and Nods (21, 22), are involved in host responses to *Chlamydia* infection and to purified *Chlamydia* components, but the role of the chlamydial MOMP had not been examined so far. Since MOMP is an integral outer membrane protein, the addition of detergents is required to preserve its native structure and to ensure solubility when it is purified from *Chlamydia* organisms. Detergent-based MOMP preparations have been used in animal immunization studies, but these cannot be successfully used *in vitro* due to the cytotoxic nature of detergents, which is amplified in the confined tissue culture well microenvironment. Thus, native purified MOMP was incorporated into detergent-free protein micelles, termed proteosomes (45), a strategy that has been successfully applied to investigate the *in vitro* and *in vivo* effects of bacterial porins. In all the cell types used in this work, no indication of cell toxicity was observed for MOMP proteosomes (not shown). In addition, the trimeric nature of MOMP was preserved, as observed by the presence of high-molecular-weight forms corresponding to trimers (and oligomers) by electrophoretic analysis (48).

Using a human nonhematopoietic cell model of TLR expression (HEK cells), we demonstrate that MOMP proteosomes in-

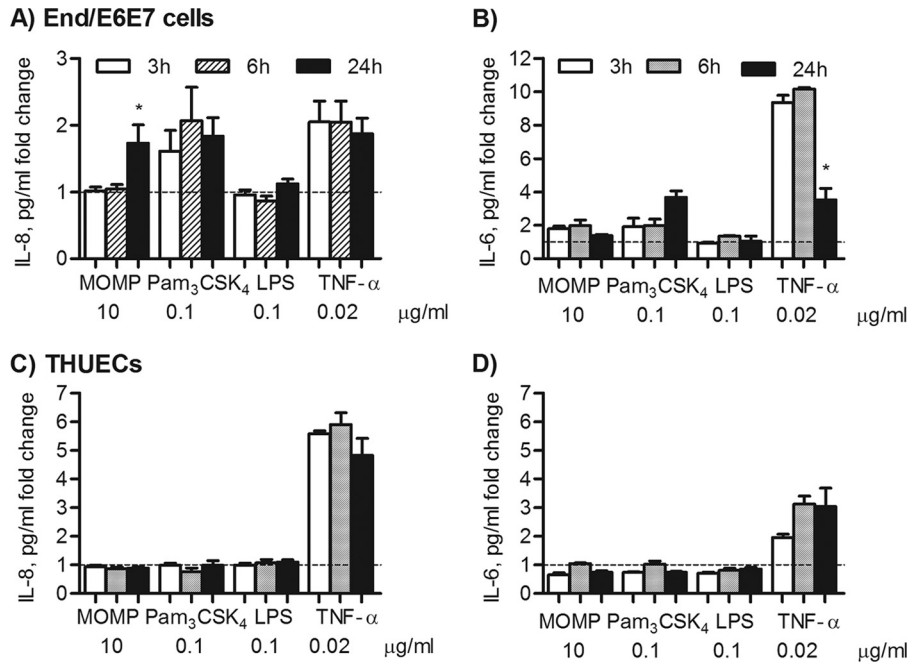


FIG 7 Time course of IL-8 and IL-6 secretion in human reproductive tract epithelial cells. Kinetics of IL-8 (A) and IL-6 (B) secretion in End/E6E stimulated with MOMP proteosomes (10 µg/ml), Pam₃CSK₄ (0.1 µg/ml), *E. coli* LPS (0.1 µg/ml), or TNF-α (0.02 µg/ml) for 3 h (white bars), 6 h (striped bars), and 24 h (black bars) (*, $P < 0.05$, by one-way ANOVA with a Bonferroni posttest; $n = 3$). IL-8 (C) and IL-6 (D) production by THUECs incubated as described above.

duce TLR2-dependent IL-8 secretion and the induction of NF-κB-dependent luciferase activity, consistent with the known TLR2-dependent activity of bacterial porins. Furthermore, TLR4 did not appear to play a role in the activity of MOMP proteosomes *in vitro*.

TLR2 signaling is dependent on the expression of coreceptors. A TLR2/TLR1 complex drives TLR2-dependent cell activation by *Neisseria* PorB, while TLR2/TLR6 mediates the activity of *Shigella* porins. Using HEK cells that overexpress the TLR2/TLR1 or TLR2/TLR6 receptor pair, we show that MOMP proteosomes induce high levels of IL-8 secretion when TLR2 and TLR1 are present on the cell surface, similar to the effect of the synthetic triacylated lipopeptide Pam₃CSK₄. In contrast, in cells that overexpress TLR2 and TLR6, MOMP proteosomes (and Pam₃CSK₄) induced low IL-8 secretion, while the diacylated lipopeptide Pam₂CSK₄ induced high levels of IL-8, consistent with TLR2/TLR6 signaling. As previously mentioned, HEK cells express low endogenous TLR1 and TLR6 levels, which can explain the background level of cell activation in response to each of the stimuli used.

Maximal expression of IL-8 is induced via combined activation of both NF-κB and AP-1 signaling pathways (via ERK1/ERK2, JNK, and p38 MAP kinases), particularly in nonhematopoietic cells (49). To dissect the intracellular signaling cascades induced by MOMP proteosomes *in vitro*, specific inhibitors of NF-κB and MAP kinase phosphorylation and activation were used. Inhibition of p38, ERK1/2, and JNK signaling, as well as NF-κB, prevented TLR2-dependent IL-8 induction by MOMP proteosomes and Pam₃CSK₄, as well as TLR2-independent cell activation by TNF-α, in agreement with multiple signaling pathways involved in TLR2-dependent (and -independent) induction of IL-8.

The use of HEK cells overexpressing TLRs is a well-established tool for the characterization of the activity of various TLR ago-

nists. However, the overabundance of TLR expression may, in part, supersede variations of activity of different agonists. For this reason, naturally TLR2-competent cells were also used to test the activity of MOMP proteosomes. Because *Chlamydia* infection is a significant public health problem, it is important to understand the mechanism(s) by which chlamydial products may activate host cell inflammatory responses at the site of colonization and infection. In the reproductive tract epithelium, as well as in other nonsterile epithelia, TLRs are selectively expressed for a tight regulation of inflammatory responses following bacterial colonization or host cell infection (56–58).

Evidence exists that TLR2 signaling may contribute to regulation of site-specific mucosal inflammation following *Chlamydia* infection, for example, more severe respiratory pathology and inflammation have been observed in TLR2 knockout mice infected with *C. muridarum* compared to wild-type mice (35). The human female epithelial reproductive tract cell line EndE6/E7 is a relevant cell model for studying the outcomes of cell stimulation with MOMP proteosomes, since *Chlamydia* infects the human reproductive tract epithelium. An additional advantage of these cells is their unresponsiveness to TLR4 agonists, due to the lack of TLR4 and MD-2 expression required for LPS signaling (51). In these cells, MOMP proteosomes induced secretion of the proinflammatory mediators IL-8 and IL-6, similar to the effect of Pam₃CSK₄ (although Pam₃CSK₄ was more potent in inducing IL-6 than MOMP proteosomes). In HeLa cells, MOMP proteosomes failed to induce both IL-8 and IL-6 secretion, possibly due to the lack of expression of TLR1 by these cells (54), which also explains the lack of responsiveness to Pam₃CSK₄ (55). Since *Chlamydia* also infects the male urethral and bladder epithelia, epithelial cells from both the female and male reproductive tracts have been used as models for *Chlamydia* infection (as well as for *Neisseria gonorrhoeae*) (47,

51). Studies in both human and rat prostate epithelial cell lines have shown that these cells express TLR4 and CD14 mRNA as well as TLR2 and TLR9 (57). It is known that, after prolonged exposure of THUECs to live *Chlamydia* organisms, IL-6, IL-1 β , and, to a lesser extent, IL-8 are induced in the cell supernatant (52). However, stimulation of THUECs with MOMP proteosomes, Pam₃CSK₄, or even *E. coli* LPS for 24 h did not induce IL-8 or IL-6 secretion, while only TNF- α stimulation led to high levels of inflammatory cytokine production.

To determine whether IL-8 and IL-6 secretion may follow different kinetics in female and male reproductive tract epithelial cells, early time points were also examined, but a time-dependent, increased IL-8 induction by MOMP proteosomes was only detected in End/E6E7. Furthermore, in End/E6E7, a reduced IL-6 secretion trend was apparent after 24 h of incubation with MOMP proteosomes and with TNF- α . From these initial observations, the speculation that MOMP could contribute to suppression of cellular inflammatory responses in TLR2-competent female reproductive tract epithelial cells during host exposure to chlamydial products may arise. It is possible that a longer stimulation with MOMP may be required to mimic the consequences of prolonged cell exposure to *Chlamydia* that would recapitulate a chronic infection. Nevertheless, our comparison of female and male reproductive tract epithelial cells may, in part, contribute to explain the differences in the inflammatory responses *in vivo* following *Chlamydia* infection, although the limitations of these *in vitro* models need to be carefully considered.

A better understanding of both the host immune responses and the bacterial immune effectors is essential for guiding new and improved approaches to control and prevent chlamydial pathogenesis. Crucial studies have suggested that TLR2-dependent host responses and murine genital tract and respiratory infections by *Chlamydia* are influenced by the presence of a cryptic plasmid in strains of *C. muridarum* and *C. trachomatis* (26), while plasmid-cured *Chlamydia* retains the ability to signal and induce pathogenesis in guinea pigs (25). However, there is no evidence that plasmid-encoded proteins are directly responsible for TLR2-mediated signaling by *Chlamydia*. In addition, since the plasmid-cured strains replicate less efficiently than the wild-type strains, a potential overall smaller amount of antigens, including MOMP, may be present in the coculture experiments *in vitro*. Whether MOMP purified from these plasmid-cured strains induces TLR2-dependent cell activation *in vitro* has not been examined. However, since the DNA sequence of the MOMP in these isolates is identical to that of the original strains, one could predict that there will be no differences in activity.

In conclusion, our results provide evidence of TLR2-dependent activity for purified MOMP proteosomes and lay the basis for investigating its potential role, as a TLR2-dependent agonist, in the induction of the inflammatory response during a chlamydial infection. Furthermore, as a vaccine candidate, it will be important to further evaluate the potential adjuvant activity of MOMP proteosomes *in vitro* and *in vivo*.

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