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Mycoplasma genitalium-Encoded MG309 Activates NF-κB via Toll-Like Receptors 2 and 6 To Elicit Proinflammatory Cytokine Secretion from Human Genital Epithelial Cells▽

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Mycoplasma genitalium **has been implicated in several important reproductive tract syndromes in women, including pelvic inflammatory disease, cervicitis, and tubal factor infertility. The mechanisms of immune activation are unclear, and we sought to determine whether *M. genitalium* was capable of activating innate immune responses through ligation of highly expressed Toll-like receptors (TLR) of the genital tract. Using HEK293 cells expressing specific human TLR, viable *M. genitalium* and the recombinant C-terminal portion of the immunogenic protein MG309 (rMG309c) were shown to activate NF-κB via TLR2/6. These data provided a putative mechanism for activation of the innate response in genital tissues. Genital epithelial cells (EC) are the first responders to sexually transmitted pathogens and express high levels of TLR2 and -6. Following exposure to purified rMG309c, vaginal and ecto- and endocervical EC secreted proinflammatory cytokines, including interleukin-6 (IL-6) and IL-8. Vaginal EC were less responsive than cervical EC. The capacity of rMG309c to bind TLR2/6 and elicit inflammation was sensitive to proteinase K digestion and independent of traditional N-terminal lipoylation. Furthermore, the immunostimulatory capacity of rMG309c was localized specifically to a 91-amino-acid subfragment of the recombinant protein, suggesting that TLR activation is likely amino acid based. Together, these data indicated that human vaginal and cervical EC are immunologically responsive to *M. genitalium* and to purified rMG309c via highly expressed TLR of the genital tract. These findings provide valuable insights into the mechanisms for activation of acute-phase inflammatory responses and suggest that *M. genitalium* colonization of reproductive tract tissues may result in inflammatory sequelae.**

Mycoplasma genitalium is a sexually transmitted pathogen (20, 27) implicated as an important cause of nongonococcal urethritis in men (28). In women, inflammatory reproductive tract disease syndromes, including pelvic inflammatory disease (17) and cervicitis (11, 24, 26, 34, 44), also have been associated with *M. genitalium* infection. However, the mechanisms for initiation of the innate immune response in these tissues have yet to be elucidated. Results presented herein establish that *M. genitalium* and the C-terminal portion of the antigenic protein encoded by MG309 activate NF-κB via Toll-like receptors 2 and 6 (TLR2/6), resulting in cytokine secretion from relevant epithelial cells (EC) of the reproductive tract. These findings elucidate a putative mechanism for induction of proinflammatory responses and provide a rationale for continued investigation into the mechanisms of *M. genitalium*-induced urogenital disease in women.

The female reproductive tract includes the normally sterile upper genital tract, composed of the uterus, fallopian tubes, and ovaries. The lower genital tract includes the polymicrobial environment of the vagina and the translational ectocervical and endocervical tissues. EC of the reproductive tract provide a physical barrier against invading pathogens and are differentially equipped as nontraditional immune cells capable of activating early innate immune responses (18, 36). These cells recognize several classes of conserved pathogen-associated molecular patterns via the Toll-like family of transmembrane receptors (TLRs). Ligation of TLRs results in activation of the innate response through secretion of proinflammatory cytokines, but no studies, to our knowledge, have addressed the genital EC response to *M. genitalium* infection.

EC of the vagina and cervix express robust levels of TLR2, -3, -5, and -6 and CD14, with low levels of TLR1, -4, and -7, -8, and -9 (19). EC are the first cells to encounter *M. genitalium* following transmission. Among TLRs implicated in recognition of bacteria, the TLR2/6 heterodimer is hypothesized to be important for recognition of bacterial ligands by reproductive EC because their expression level and immunologic responsiveness are increased relative to other TLRs (19). Vaginal and cervical EC respond to specific TLR2/6 agonists with robust proinflammatory cytokine elaboration, including the *Mycoplasma fermentans* lipopeptide (13) and the fibroblast-stimulating lipopeptide 1 of *Mycoplasma salivarium* (FSL-1) (19). In concordance, the immortalized human vaginal, ectocervical, and endocervical EC characterized previously in our laboratory (19) were used to evaluate the inflammatory capacity of the antigenic MG309 protein encoded by *M. genitalium*.

It is well-documented that lipoproteins from select pathogenic *Mycoplasma* spp. can interact with TLR, leading to in-

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flammatory cytokine production. Like other *Mollicutes*, *M. genitalium* contains a significant proportion of lipoproteins in the cell membrane (45), consistent with the identification of 21 putative lipoprotein genes in the 580-kb genome (15). It has been shown recently that Triton X-114 preparations of detergent-soluble components from *M. genitalium* upregulate transcription of tumor necrosis factor alpha, interleukin-1β (IL-1β), and IL-6 mRNA in the human THP-1 monocytic cell line (46, 47), suggesting that lipoproteins may, at least in part, be responsible for immune activation. Of the 21 putative lipoprotein genes, we selected MG309 for inflammatory characterization because it is expressed during *M. genitalium* infection and is recognized by sera of *M. genitalium* patients, suggesting immune recognition by the host (L. Ma and D. H. Martin, unpublished findings). Interestingly, a specific region within the MG309 coding sequence has been shown previously to have significant variability in the number of short tandem repeats (STR) among and within clinical isolates (25). This suggests that the STR region of this gene may be under immune-mediated selective pressure in *M. genitalium*-infected patients.

Our results establish that the intact *M. genitalium* organism and a localized region within the C terminus of the MG309-encoded protein (amino acids 919 to 1009) activate NF-κB via TLR2/6 heterodimers, ultimately leading to proinflammatory cytokine elaboration from human vaginal and ecto- and endocervical EC. These findings establish a putative TLR2/6-mediated mechanism for induction of inflammatory reproductive tract disease in *M. genitalium* patients and elucidate a specific immunogenic protein capable of activating the innate immune response.

MATERIALS AND METHODS

Propagation of *M. genitalium* G37. *M. genitalium* type strain G37 (ATCC 33530) was propagated in modified Friis FB medium (22). Briefly, frozen (−80°C) mycoplasma stocks were inoculated into 10 ml of freshly prepared Friis medium in tightly sealed tissue culture flasks and incubated at 37°C for 5 to 8 days. Growth was monitored by the formation of adherent microcolonies and a pH-mediated color change of the medium. *M. genitalium* was harvested from culture flasks by pouring off the spent medium, washing adherent mycoplasma cells five times with a total volume of approximately 15 to 20 ml of sterile phosphate-buffered saline (PBS), and then scraping adherent mycoplasma cells into fresh PBS. Quantification of *M. genitalium* viability was done in 96-well plates by serial 10-fold dilution of each sample into fresh Friis medium. The last dilution to show a change in color was used to calculate the number of viable organisms in the original sample. Heat denaturation of *M. genitalium* G37 was accomplished by incubating log-phase cultures at 80°C for 15 min and then cooling on ice. Loss of *M. genitalium* viability was verified by an absence of growth in Friis medium after 14 days of incubation at 37°C.

Human cell culture. Immortalized human EC derived from vaginal, ectocervical, and endocervical tissues were maintained as described previously (19). Briefly, ectocervical and endocervical EC were maintained in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract (50 mg/liter), recombinant epidermal growth factor (5 μg/liter), $CaCl_2$ (44.1 mg/liter), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 humidified incubator (36). Vaginal EC were maintained in a 1:1 mixture of keratinocyte serum-free medium and VEC-100 medium (MatTek, Ashland, MA). Human embryonic kidney cells stably transfected with selected human TLR (HEK293; InvivoGen) (16) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), 0.1 mM nonessential amino acids (Sigma-Aldrich, St. Louis, MO), and 2 mM L-glutamine (Invitrogen).

Cloning and purification of recombinant MG309 (rMG309c). Mycoplasmas utilize the UGA codon to encode tryptophan rather than a termination codon, making it difficult to express mycoplasma genes in *Escherichia coli* hosts. The MG309 gene from *M. genitalium* G37 (bp 382732 to 386409; GenBank accession number L43967) contains seven UGA codons (see Fig. 2, below). The largest fragment of MG309 free of UGA stop codons that corresponded to the C terminus (bp 385488 to 386397; amino acids 919 to 1222) was amplified from *M. genitalium* G37 genomic DNA by PCR using primers 5′-GGATCCACCAACA ACAAAACCCCAACAG-3′ and 5′-GTCGACGCTGTGAACTGATTGCTG A-3′, which contained BamHI and SalI restriction sites (underlined), respectively, added to the 5′ ends. To determine specific proinflammatory regions of the MG309 gene, three subfragments of cloned recombinant MG309 (rMG309c) referred to as the N- (amino), M- (middle), and C- (carboxyl) constructs rMG309c-N, rMG309c-M, and rMG309c-C, respectively, were amplified with primers containing BamHI and SalI restriction sites corresponding to amino acids 919 to 1009 (rMG309c-N, bp 385488 to 385760, 5′-GGATCCACCAACA ACAAAACC-3′ and 5′-CGTCGACCCACCACTTGATTGAGTAGA-3′)], amino acids 1010 to 1114 (rMG309c-M, bp 385761 to 386076, 5′-GGATCCTC TACTCAATCAAGTGG-3′ and 5′-CGTCGACCCATTTGTACCTATATACC C-3′) and amino acids 1115 to 1222 (rMG309c-C, bp 386077 to 386396, 5′-GG ATCCGGGTATATAGGTACAAAT-3′ and 5′-CGTCGACCCGAATTTACG CACC-3′). Each PCR product was cloned into the pPROEX-HTb expression plasmid (Invitrogen), which directs expression of the recombinant protein with a six-histidine tag, allowing purification using a nickel affinity column. The empty pPROEX-HTb vector and the *Salmonella enterica* serovar Dublin flagellin (FLAG) *fliC* gene (10) in pPROEX-HTb were used as controls. Plasmid constructs were confirmed by restriction digest and DNA sequencing and then expressed in *E. coli* BL21(DE3) (Protein Express, Cincinnati, OH). Expression of rMG309c, subfragments of rMG309c, or FLAG was induced using isopropyl β-D-1-thiogalactopyranoside (0.1 M). The optimum duration of expression was determined by kinetic evaluation of induced *E. coli* lysates using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of six-His fusion proteins was accomplished first by lysozyme-mediated lysis and sonication of induced *E. coli* cell pellets. Cell lysates then were applied to Ni^{2+}-charged resins and purified under denaturing conditions with 6 M urea (His-Bind purification; Novagen, Darmstadt, Germany). Purified proteins were dialyzed overnight at 4°C in 3 liters of PBS to remove urea and any free lipopolysaccharide (LPS). The correct size and purity of rMG309c preparations were verified using SDS-PAGE with Coomassie staining and reactivity with a polyclonal rabbit serum against an *M. genitalium* G37 whole-cell lysate. The yield of recombinant protein was measured using the protein quantification assay (Bio-Rad Laboratories, Hercules, CA). Contaminating *E. coli* endotoxin was quantified using the Pyrotell *Limulus* amebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA). Heat denaturation was performed by incubating purified rMG309c preparations (0.5 μg/ml) at 95°C for 15 min and then cooling on ice. Proteinase K digestion was done by mixing 3 μl proteinase K solution (Qiagen, Valencia, CA) with 20 μl of the stock recombinant protein preparation and incubation at 56°C for 1 h. Digestion of rMG309c was verified by SDS-PAGE. Following proteinase K treatment, the digested sample was heat inactivated as described above to ensure no protease activity was carried forward into the TLR or cytokine assays.

TLR stimulating compounds. The synthetic lipopeptide Pam2-CGDPKH PKSF (FSL-1), derived from *Mycoplasma salivarium* (InvivoGen, San Diego, CA), was used as a TLR2 and TLR2/6 agonist for the HEK293 cell studies and for quantification of cytokine secretion from genital EC (0.1 μg/well in a 96-well plate). Recombinant endotoxin-free *S. enterica* serovar Dublin flagellin (FLAG; 1 ng/well in a 96-well plate) (10), provided by T. Eaves-Pyles, was used as a positive control for TLR5-dependent NF-κB activation. *E. coli* LPS (Sigma-Aldrich) was applied to HEK293 cells expressing human TLR4/CD14 as a positive control for NF-κB activation and to establish the minimal concentration required for cytokine elaboration from genital EC.

NF-κB reporter system for TLR-mediated responsiveness. HEK293 cells stably expressing TLR2, TLR2/6, TLR4/CD14, or TLR5 (InvivoGen) (16) were seeded into T-75 culture flasks and incubated overnight at 37°C in an atmosphere containing 5% CO_2. Cells then were transfected with the pNF-κB-secreted alkaline phosphatase (pNF-κB-SEAP) reporter plasmid (Clontech, Mountain View, CA) using Lipofectamine Plus (Invitrogen) per the manufacturer's instructions. Transfected cells were seeded into 96-well plates (5×10^4/well) and incubated overnight. Purified rMG309c, rMG309c subfragments (10 ng to 1 μg/well), FSL-1 (0.1 μg/well), *S. enterica* serovar Dublin FLAG (1 ng/well), or viable *M. genitalium* G37 cells (multiplicity of infection [MOI], 1 to 100) were applied to triplicate wells of HEK293-hTLR cells and incubated for 24 h. Supernatants were removed and heat inactivated for 30 min at 65°C to destroy endogenous alkaline phosphatases. SEAP activity, directly representing NF-κB activation, was quantified using the Phospha-Light chemiluminescence reporter assay (Tropix, Bedford, MA) and a TR717 microplate luminometer with Win-

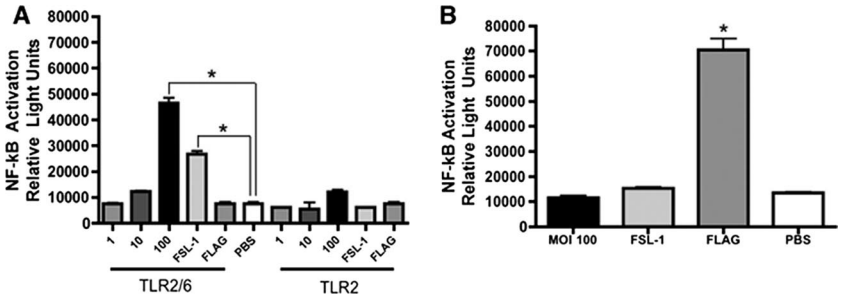

FIG. 1. *M. genitalium* G37 induced TLR2/6-mediated NF-κB activation. HEK293 cells, stably expressing human TLR2/6, TLR2 (A), or TLR5 (B) were transfected with an NF-κB-responsive SEAP reporter plasmid to quantify NF-κB activation in response to *M. genitalium* exposure. Extensively washed viable *M. genitalium* cells (MOI, 1 to 100) were applied to HEK293 cells, and then culture supernatants were collected 24 h postincubation for SEAP quantification. Purified recombinant *S. enterica* serovar Dublin FLAG and FSL-1 from *M. salivarium* were used as TLR specificity controls and processed in parallel on each cell type. Data shown are the means ± standard errors of the means of SEAP induction collected in two independent experiments performed in triplicate wells. Statistical comparisons were made using one-way ANOVA followed by Dunnett's post test. ∗, $P < 0.01$ versus PBS vehicle control.

Glow software (Tropix/PE Applied Biosystems, Bedford, MA). Relative fluorescent unit values were normalized by calculating the mean SEAP secretion from cells expressing TLR2/6 treated with the PBS vehicle and then adjusting the data set based on the other cell types to facilitate a comparison of relative responsiveness.

Stimulation of human genital EC. Human vaginal, ectocervical, or endocervical EC were seeded at 1×10^5 cells/well. Following overnight incubation at 37°C, culture supernatants were removed and replaced with fresh medium containing purified rMG309c or subfragments of rMG309c (0.5 μg/well). Each experimental condition was tested in triplicate wells. Positive controls for TLR-mediated stimulation included FSL-1 (0.1 μg/well) and *E. coli* LPS (100 ng to 1 pg/well). As a negative control for experimental manipulation, an equal volume of the PBS vehicle was added to triplicate wells and processed in parallel to establish baseline cytokine levels. Following 6 h of incubation at 37°C, secreted cytokines were quantified from culture supernatants via cytometric bead array assay using the human 27-plex panel of cytokine targets (Bio-Rad Laboratories).

Statistical analyses. A one-way analysis of variance (ANOVA) followed by Dunnett's post test (Prism v. 4.0; GraphPad, San Diego, CA) was used to calculate significant differences in SEAP activity or cytokine secretion levels when more than two conditions were compared. The Student's *t* test was used for comparisons of differences in magnitude for individual cytokines. Significance was indicated when P was <0.05.

RESULTS

***M. genitalium* G37 activated NF-κB via TLR2/6.** Activation of NF-κB is a key step in TLR-mediated responses and is necessary to induce the innate immune response to pathogens ultimately resulting in inflammatory cytokine production (6). Mechanistically, *M. genitalium* was predicted to ligate TLRs that are expressed at high levels in genital EC via antigenic proteins such as MG309. Consistent with this hypothesis, viable *M. genitalium* elicited significant NF-κB activation in HEK293 cells expressing TLR2/6 at an MOI of 100 relative to PBS alone ($P < 0.01$) (Fig. 1A). In cells expressing TLR2 alone, viable *M. genitalium* elicited low NF-κB activation at an MOI of 100, but this increase was not significant ($P > 0.05$). Using HEK293 cells expressing hTLR5, no significant increase in NF-κB activation was observed relative to PBS alone in response to *M. genitalium* G37, but these cells were highly responsive to purified recombinant FLAG ($P < 0.01$ versus PBS vehicle control) (Fig. 1B).

Construction and purification of endotoxin-free rMG309c. We cloned the largest MG309 fragment free of UGA stop codons, which corresponded to the C-terminal region (bp 385488 to 386397). The resulting recombinant protein (rMG309c) was expressed to high levels in *E. coli*. MG309 clones were verified by DNA sequencing. Purified rMG309c (Fig. 2A) was present as a single band of approximately 40 kDa on SDS-PAGE gels (Fig. 2B, lane 1). This size was consistent with the predicted molecular mass calculated from the cloned DNA sequence. Stock preparations of purified rMG309c were recognized by a rabbit polyclonal antiserum raised to *M. genitalium* lysates (Fig. 2B, lane 2) and by

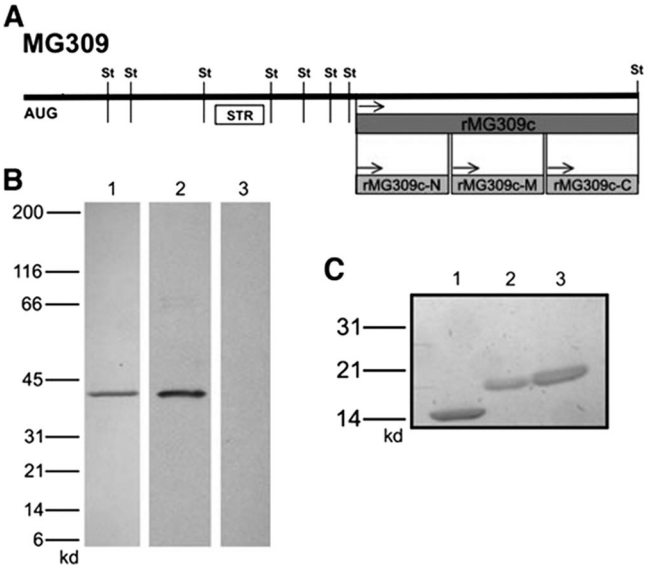

FIG. 2. Purified rMG309c induced proinflammatory cytokine elaboration. (A) The largest coding fragment of MG309 (designated rMG309c) free of UGA codons (stop signal [St] in *E. coli*) was PCR amplified and cloned into pPROEX-HTb for direct expression of a six-His-tagged recombinant protein in *E. coli* BL21 cells. The chosen coding region did not include the highly variable STR stretch, which varies significantly in length within in vitro and clinical specimens. (B) Nickel-purified rMG309c ran at approximately 40 kDa on a SDS-PAGE gel (25 μg load; Coomassie stain, lane 1) and was recognized by a rabbit polyclonal antibody generated against *M. genitalium* G37 lysates (lane 2) but not by preimmune serum (lane 3). (C) Three subfragments of rMG309c representing the N terminus (rMG309c-N; approximately 15 kDa), middle (rMG309c-M; approximately 18 kDa), and carboxyl terminus (rMG309c-C; approximately 18 kDa) were purified similarly and run on SDS-PAGE gels (lanes 1, 2, and 3, respectively; Coomassie stain).

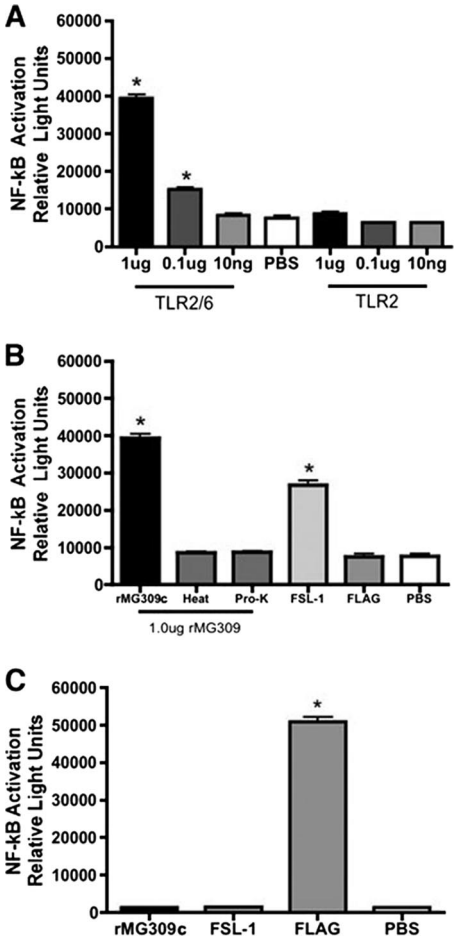

FIG. 3. rMG309c induced TLR2/6-mediated NF-κB activation. HEK293 cells, stably expressing TLR2/6 (A and B), TLR2 (A), or TLR5 (C) were transfected with a NF-κB-responsive SEAP reporter plasmid to quantify NF-κB activation. (A) Purified rMG309c was applied to each HEK293 cell type (1 μg to 10 ng/well), and then culture supernatants were collected 24 h later for quantification of SEAP. (B) The sensitivity of rMG309c to proteinase K digestion or heat denaturation was tested on HEK293 cells expressing TLR2/6. An equivalent molar amount of purified recombinant FLAG or FSL-1 was processed in parallel on TLR2/6- (B) or TLR5-expressing cells (C) as a control for TLR specificity. Data shown are the normalized means ± standard errors of the means of SEAP induction collected in two independent experiments performed in triplicate wells. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's post test. *, $P < 0.01$ versus PBS vehicle control.

M. genitalium-infected patient sera (data not shown). This protein was not recognized by preimmune serum from the same rabbit (Fig. 2B, lane 3). The three subfragments of the C-terminal MG309 peptide corresponding to the N terminus (rMG309-N; predicted, 15 kDa), middle (rMG309-M; predicted, 18 kDa), and C terminus (rMG309-C; predicted, 18 kDa) were expressed and purified to similar concentrations and purities (Fig. 2C). The amount of LPS endotoxin in undiluted recombinant protein preparations was quantified using the *Limulus* amebocyte assay and was consistently less than 10 pg/ml. This concentration was below the minimum stimulatory concentration for HEK293 cells expressing TLR4/CD14 or genital EC. Furthermore, HEK293 cells expressing TLR4 and CD14 were not responsive to rMG309c, indicating a lack of LPS contamination (data not shown).

rMG309c activated NF-κB via TLR2/6. Purified rMG309c (1 μg and 0.1 μg/well) elicited significant NF-κB activation in HEK293 cells expressing human TLR2/6 compared to PBS ($P < 0.01$) but not in cells expressing TLR2 alone (Fig. 3A). Heat denaturation and proteinase K digestion of purified rMG309c reduced the SEAP production to baseline levels in cells expressing TLR2/6 (Fig. 3B). An equimolar quantity of FSL-1 (0.1 μg/well) induced significant SEAP production in cells expressing TLR2/6 (Fig. 3B) ($P < 0.01$). As expected, cells expressing TLR2/6 were not responsive to the purified recombinant TLR5 agonist, FLAG from *S. enterica* serovar Dublin (1 ng/well) (Fig. 3B). HEK293 cells expressing human TLR5 (Fig. 3C) or TLR4/CD14 (data not shown) did not respond to rMG309c (1 μg/well) or FSL-1 and served as a control for TLR specificity.

rMG309c elicited cytokine secretion from human genital EC. EC of the female reproductive mucosa express TLR1 to -9 (12, 35, 39) but express the highest levels of TLR2, -3, -5, and -6 (19). To determine whether rMG309c was immunostimulatory to female genital tract EC, purified rMG309c was applied to immortalized human vaginal and ecto- and endocervical EC to quantify induced cytokine responses. rMG309c elicited significant levels of IL-8 secretion that peaked 6 h after application to each EC type ($P < 0.05$) (Table 1). Secretion of granulocyte colony-stimulating factor (G-CSF) and granulocyte-monocyte (GM)-CSF was significantly increased compared to PBS controls from both ecto- and endocervical EC but not vaginal EC. Finally, endocervical EC, but not vaginal or ectocervical EC, exposed to rMG309c also secreted significant levels of IL-6 ($P < 0.05$) (Table 1). As observed with the HEK293 cells, heat denaturation or proteinase K digestion

TABLE 1. Cytokine elaboration from human vaginal and endo- and ectocervical EC following exposure to purified rMG309c[a]

Cytokine	Mean cytokine concn (pg/ml) from:					
	Vaginal EC		Ectocervical EC		Endocervical EC	
	rMG309c	PBS	rMG309c	PBS	rMG309c	PBS
IL-6	34.8 ± 5.1	27 ± 2.6	27.1 ± 3.2	14.2 ± 0.63	114 ± 3.1*	26.6 ± 2.9
IL-8	186 ± 0.6*	132 ± 11	1,792 ± 38*	167 ± 17	62,997 ± 6,667*	3,705 ± 1,440
G-CSF	17 ± 0.5	12 ± 0.1	368 ± 7.5*	181 ± 3.2	200 ± 5.1*	24 ± 2.4
GM-CSF	<LL	<LL	10.8 ± 0.9*	4.9 ± 0.4	32 ± 4.0*	6 ± 1.9

[a] Vaginal EC and ecto- and endocervical EC were stimulated with rMG309c (0.5 μg/well) or an equal volume of the PBS vehicle as a control. Culture supernatants were collected 6 h after stimulation to quantify secreted cytokines as described in Materials and Methods. Values are expressed as means ± standard errors of the means. <LL (less than the lower limit) indicates that values were too low to be calculated from the standard curve. *, significantly increased compared to PBS vehicle control ($P < 0.05$; Student's *t* test).

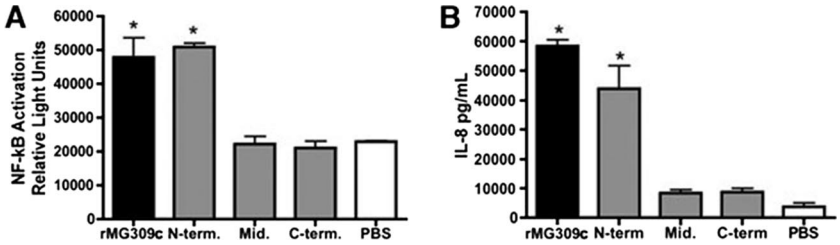

FIG. 4. TLR2/6 binding and inflammatory capacities of rMG309c were localized to a 91-amino-acid region. (A) HEK293 cells, stably expressing TLR2/6, were transfected with an NF-κB-responsive SEAP reporter plasmid to quantify NF-κB activation. Three subfragments of rMG309c were purified and tested for their abilities to activate NF-κB via TLR2/6. (B) To quantify the cytokine response to the rMG309c subfragments, equimolar amounts of purified rMG309c or the three subfragments were applied to human vaginal and ecto- and endocervical EC. Cytokines were quantified from culture supernatants 6 h later. Data shown are the means ± standard errors of the means from a representative study that was replicated in two independent experiments performed in triplicate wells. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's post test. *, $P < 0.01$ versus PBS vehicle control.

significantly reduced the inflammatory capacity of rMG309c to levels equivalent to the PBS vehicle (data not shown).

The inflammatory capacity of rMG309c was localized to a 91-amino-acid region. Based on the absence of the native N terminus and traditional lipoylation, we tested the hypothesis that specific regions within the C-terminal rMG309c peptide were responsible for TLR2/6 activation. Three rMG309c subfragments (Fig. 2A and C) were cloned, purified, and then tested for TLR2/6 activation. Application of equimolar amounts of each subfragment showed that the TLR2/6-stimulating capacity was localized to amino acids 919 to 1009, comprising the N terminus of the rMG309 protein (rMG309c-N) (Fig. 4A). This 91-amino-acid region also elicited significant proinflammatory IL-8 elaboration from endocervical EC (Fig. 4B), the most sensitive genital EC type in our studies. The IL-8 levels were similar in magnitude to those with the complete rMG309c peptide (Table 1). The pattern of tested cytokines induced by the N-terminal subfragment was identical to that with the full-length rMG309c in vaginal and ecto- and endocervical EC cultures (data not shown). NF-κB activation and induction of inflammatory cytokine secretion by the N-terminal region of rMG309c were equivalent to the induction from the complete rMG309c protein, indicating the TLR2/6-activating capacity was specifically localized to this region. No TLR2/6-mediated NF-κB activation or proinflammatory cytokine secretion from vaginal or cervical EC was observed from the middle or C-terminal rMG309c subfragments (Fig. 4), thereby confirming the specific activity of the N-terminal region within the C terminus of MG309.

DISCUSSION

M. genitalium infection of the reproductive tissues in women is associated with cervicitis (11, 24, 26, 34, 44), endometritis (8), pelvic inflammatory disease (17) and, with less conclusive evidence, tubal factor infertility (7, 42). The mechanisms and cell types involved in the early recognition of *M. genitalium* infection and activation of the innate immune response remain unclear. We investigated whether *M. genitalium* G37 was recognized by highly expressed TLRs throughout the female reproductive tract and verified these findings using a specific recombinant antigenic protein, MG309.

We observed significant activation of NF-κB in response to viable *M. genitalium* via TLR2/6 heterodimers expressed in HEK293 cells (Fig. 1A). NF-κB activation was observed via TLR2 alone but at a significantly reduced level and only at the highest concentration of *M. genitalium*, suggesting that TLR2 homodimers likely are not sufficient for robust recognition of *M. genitalium*. Interaction of *M. genitalium* with cell surface TLRs, such as TLR2 and -6, is a plausible mechanism for innate immune response activation, as clinically isolated *M. genitalium* strains have been shown to directly interact with the host cell surface during the process of adhesion and entry into host cells (21, 29). Activation of TLR2/6 by the intact *M. genitalium* organism suggested that the membrane-associated proteins likely mediate a significant proportion of the innate immune activation from reproductive tract tissues, as TLR2 is involved commonly in recognition of bacterial proteins (43).

Mycoplasmas have no cell wall but contain a single plasma membrane system (3, 38) comprised heavily of proteins (37, 45) that are associated with the ability to adhere to the epithelium (2, 14). Considering the observed antigenic characteristics of MG309, it was hypothesized that MG309 might also activate innate immune responses via TLR2/6. MG309 was targeted for investigation because it is expressed during genital tract infection and axenic culture (25). A homolog of MG309 exists in *Mycoplasma pneumoniae* (MPN444), but no studies to our knowledge have addressed antigenicity or a role in inflammation. Similar to the intact *M. genitalium* organism, significant activation of NF-κB was observed via TLR2/6 heterodimers but not TLR2 alone (Fig. 3A). A recent report showing that MG149 activates NF-κB via TLR1/2 (41) further supports the hypothesis that *M. genitalium* is capable of eliciting innate immune responses via cell surface TLR. However, TLR1 expression in vaginal and cervical EC is very low (19), so we did not evaluate whether rMG309c is capable of activating TLR1/2 heterodimers. Importantly, MG309 is likely one of several *M. genitalium* proteins capable of inducing proinflammatory cytokines following *M. genitalium* exposure. In fact, other highly expressed antigenic lipoproteins, including MgPa, are putative agonists for cellular TLR and currently are under investigation for their role in immune stimulation and antigenic variation.

We further investigated the ability of rMG309c to elicit proinflammatory innate responses from vaginal and cervical EC. Immortalized human vaginal, ectocervical, and endocer-

vical EC responded to rMG309c with low, but significant, elaboration of proinflammatory cytokines (Table 1). The pattern of acute-phase cytokines induced by rMG309c in these cells was largely similar to that induced by FSL-1, an *M. salivarium*-derived lipopeptide known to specifically ligate TLR2/6 (19). Importantly, the pattern was consistent with immune cell recruitment (1, 23) and was very similar to EC responses to the intact *M. genitalium* organism (C. L. McGowin, V. L. Popov, and R. B. Pyles, submitted for publication). The robust secretion of IL-8, IL-6, G-CSF, and GM-CSF, most notably from endocervical EC (Table 1), strongly suggested that monocytes and macrophages might be recruited to infected reproductive tract tissues. These findings are important for predicting the capacity of *M. genitalium* to elicit inflammation in upper reproductive tract tissues and warrant continued investigation at these sites. Collectively, these findings suggest that TLR2/6 ligation by MG309 likely is an important mechanism for activation of the innate response to *M. genitalium* in the reproductive tract.

The focus of this work was to elucidate a putative mechanism for the observed inflammatory responses using the intact *M. genitalium* organism and a representative antigenic protein, MG309. Therefore, although MG309 encodes a putative lipoprotein with a predicted N-terminal transmembrane domain, an extensive analysis of the lipoylation state of rMG309c has not been completed. Furthermore, the native N terminus of MG309 was not included in the regions we investigated and thus it was speculated that the TLR2/6-activating capacity was independent of lipoylation and likely amino acid based. The conclusion that rMG309c activated TLR2/6 independently of N-terminal lipoylation was supported strongly by the ablation of intact *M. genitalium* and rMG309c's TLR2/6-binding capacity following proteinase K digestion and heat denaturation (Fig. 3B). In contrast, the macrophage-stimulating capacities of enriched lipoproteins from cultured *Mycoplasma* species *M. fermentans* (30), *M. hyorhinis* (31), *M. salivarium* (40), and *M. arthritidis* (9) were not significantly affected by proteinase K digestion. However, enriched detergent-phase proteins from *M. hominis* (32) and *U. urealyticum* (33) were sensitive to proteinase K digestion, suggesting that some genital mycoplasmas may encode proteins capable of activating inflammatory cytokine elaboration independent of lipidation.

It was demonstrated previously that the human TLR2/6 specificity of mycoplasmal lipoproteins is not solely dependent upon N-terminal lipoylation but also on the amino acid composition of the C and N termini and three-dimensional protein folding (4, 5). To determine which region of rMG309c was activating TLR2/6, we cloned and purified three subfragments of rMG309c to test the TLR2/6-stimulating capacity of each fragment independently. It was discovered that the 91 amino acids composing the N-terminal region of rMG309c were solely responsible for the TLR2/6-activating capacity. Furthermore, only the N-terminal subfragment was capable of eliciting proinflammatory cytokine elaboration from endocervical EC with levels and patterns equivalent to the full-length rMG309c. It is likely that the specific region responsible for TLR ligation is a smaller polypeptide sequence, but these studies are not yet complete. It is important to note that, although these data are quite convincing, we analyzed roughly half of the full-length MG309-encoded protein, and therefore some inhibitory or additional stimulatory activity may be associated with the region we did not investigate. Therefore, although the TLR2/6 mechanism for immune stimulation is supported both by rMG309c and the viable organism, the magnitude of the observed responses may not accurately represent the inflammatory capacity of the native protein.

Collectively, our results indicate that *M. genitalium* is capable of eliciting early innate immune responses, including NF-κB activation via TLR2/6. Similarly, the antigenic protein MG309 was shown to activate TLR2/6, ultimately resulting in immune activation and proinflammatory cytokine elaboration from relevant EC types of the vagina and cervix. Considering the high expression levels of TLR2 and -6 in EC of the reproductive tract, and because *M. genitalium* is capable of attachment to and invasion of the genital EC used in our studies (McGowin et al., submitted), it seems possible that acute and persistent colonization of genital tissues by *M. genitalium* may indeed result in prolonged inflammation of infected tissues. Continued investigation into the specific mechanisms of TLR-mediated activation of the innate immune response is ongoing and will be important to better understand the capacity of *M. genitalium* to cause urogenital disease in women.

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