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# Response to *Neisseria gonorrhoeae* by Cervicovaginal Epithelial Cells Occurs in the Absence of Toll-Like Receptor 4-Mediated Signaling<sup>1</sup>

Raina N. Fichorova,\* Amanda O. Cronin,<sup>†</sup> Egil Lien,<sup>‡</sup> Deborah J. Anderson,\* and Robin R. Ingalls<sup>2†</sup>

Toll-like receptors (TLRs) have recently been identified as fundamental components of the innate immune response to bacterial pathogens. We investigated the role of TLR signaling in immune defense of the mucosal epithelial cells of the lower female genital tract. This site provides first line defense against microbial pathogens while remaining tolerant to a complex biosystem of resident microbiota. Epithelial cells derived from normal human vagina, ectocervix, and endocervix expressed mRNA for TLR1, -2, -3, -5, and -6. However, they failed to express TLR4 as well as MD2, two essential components of the receptor complex for LPS in phagocytes and endothelial cells. Consistent with this, endocervical epithelial cells were unresponsive to protein-free preparations of lipooligosaccharide from *Neisseria gonorrhoeae* and LPS from *Escherichia coli*. However, they were capable of responding to whole Gram-negative bacteria and bacterial lysates, as demonstrated by NF- $\kappa$ B activation and proinflammatory cytokine production. The presence of soluble CD14, a high-affinity receptor for LPS and other bacterial ligands, enhanced the sensitivity of genital tract epithelial cells to both low and high concentrations of bacteria, suggesting that soluble CD14 can act as a coreceptor for non-TLR4 ligands. These data demonstrate that the response to *N. gonorrhoeae* and other Gram-negative bacteria at the mucosal surface of the female genital tract occurs in the absence of endotoxin recognition and TLR4-mediated signaling. *The Journal of Immunology*, 2002, 168: 2424–2432.

The mucosal surface of the lower female genital tract is a complex biosystem, providing a barrier against the outside world and participating in both innate and acquired immune defense. This mucosal compartment has adapted to a dynamic nonsterile environment challenged by a variety of antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota. Thus, the epithelial cells, lymphocytes, macrophages, and dendritic cells associated with the genital tract have unique features that enable them to adapt to this dynamic milieu. This would include the expression of hormone receptors, hormone-dependent immune function, and unique genital tract-specific defensins and mucins (1–5).

The epithelial cells that line mucosal surfaces are often the first cells to contact microbial pathogens, as normally there are very few immune cells present in the cervicovaginal mucosa and lumen (6, 7). Here, they initiate and coordinate the inflammatory response, alerting adjacent epithelium and the underlying immune cells of the potential danger posed by various microorganisms.

Despite the important role that the genital tract mucosa plays in immune defense, little is known about the mechanism of epithelial cell activation by pathogens and the receptors and secondary mediators involved in this regulated response. We have recently established an in vitro model to study epithelial-microbial interaction in this compartment using immortalized epithelial cells derived from human vagina, ectocervix, and endocervix. These cells maintain the normal immunobiological characteristics of their tissues of origin and differ extensively from commonly used tumorigenic cell lines, such as HeLa cells (8, 9). We have used this model to study the downstream effects of cell signaling following *Neisseria gonorrhoeae* infection. Our previous investigations showed that all three epithelial cell lines respond to *N. gonorrhoeae* by up-regulation of cytokines and adhesion molecules and that these responses are independent of bacterial internalization and IL-1 production (10). However, the key components of the signaling pathways involved in the inflammatory responses of cervicovaginal epithelial cells to Gram-negative bacteria have yet to be identified.

In this study we determined the expression profiles of members of the Toll-like receptor (TLR)<sup>3</sup> family in human cervicovaginal epithelial cells and correlated this with proinflammatory responses to bacterial pathogens. The TLR family plays a pivotal role in the recognition of bacterial ligands by the innate immune system (11, 12). Toll was initially identified as a receptor involved in embryonic development, where it controls dorsoventral polarization (13). It was later demonstrated that Toll and the related molecule, 18-Wheeler, control important antimicrobial responses against both fungi and bacteria in the adult fly (14). At least 10 mammalian

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<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; HKLM, heat-killed *Listeria monocytogenes*; LBP, LPS binding protein; LOS, lipooligosaccharide; sCD14, soluble CD14; sMALP-2, synthetic lipopeptide based on the full-length MALP-2; SPG, soluble peptidoglycan.

orthologs of Toll, designated TLRs, have been identified, and several of them have been implicated in cellular responses to bacterial pathogens. While both TLR2 and TLR4 were initially implicated in cellular responses to LPS, the overwhelming evidence to date suggests that these two receptors have different roles in the recognition of pathogens. TLR4 is required for recognition of LPS (also known as endotoxin), the most proinflammatory cell wall component of Gram-negative bacteria (15–19). A small secreted protein, known as MD2, which associates with TLR4 on the cell surface of innate immune cells, may also be required for TLR4-mediated responses to LPS (20, 21). TLR2 has a broader role as a pattern recognition receptor for a variety of microbes and Gram-positive ligands, e.g., bacterial lipopeptides, lipoprotein, lipoteichoic acid, and peptidoglycan (22–29). There is also some evidence that TLR2 may require association with TLR6 for recognition of certain ligands, including some lipoproteins (30, 31), peptidoglycan (32), and phenol-soluble modulins (33). In addition, TLR9 has been associated with responses to bacterial DNA (34), and TLR5 has been associated with responses to bacterial flagellin (35).

A key component of both TLR and IL-1 signaling is the adapter molecule MyD88. MyD88 is a unique protein that has homology to the cytoplasmic domain of the IL-1 and Toll receptors at the C-terminal, and an N-terminal death domain (36, 37). It is recruited by the IL-1 or Toll receptor complex after receptor stimulation and is an essential step in the sequential recruitment of the downstream kinases IL-1R-associated kinase and TNFR-associated factor-6, activation of NF- $\kappa$ B, and transcription of a myriad of proinflammatory molecules (38, 39).

We found that cervical and vaginal epithelial cells expressed mRNA for TLR1, -2, -3, -5, and -6, but failed to express TLR4 and MD2, two essential components of the receptor complex for responses to LPS. Consistent with this, cervical epithelial cells failed to respond to protein-free preparations of endotoxin derived from enteric (*Escherichia coli*) and nonenteric (*N. gonorrhoeae*) Gram-negative species, as measured by cytokine production and NF- $\kappa$ B activation. In contrast, the cells remained sensitive, in a MyD88-dependent fashion, to TLR2 bacterial ligands, i.e., lipoproteins and peptidoglycan, as well as to bacterial lysates and infection with whole *N. gonorrhoeae*. Interestingly, although cervicovaginal cells lack endogenous membrane CD14, soluble CD14 (sCD14), which was found in semen and cervicovaginal secretions, enhanced their proinflammatory host response to *N. gonorrhoeae*. These findings suggest that the lower female genital tract responds to Gram-negative bacterial components in the absence of endotoxin recognition and TLR4-mediated signaling.

## Materials and Methods

### Reagents

PBS and trypsin-versene mixture (trypsin-EDTA) were obtained from BioWhittaker (Walkersville, MD), and FCS (<10 pg/ml LPS) was purchased from Summit Biotechnology (Greeley, CO). Recombinant human IL-1 $\beta$  and TNF- $\alpha$  were purchased from R&D Systems (Minneapolis, MN). Recombinant human sCD14 and LPS binding protein (LBP) were gifts from H. Lichtenstein (Amgen, Thousand Oaks, CA). Lipo-oligosaccharide (LOS) purified from *N. gonorrhoeae* (strain 1291) was a gift from N. Qureshi (University of Wisconsin, Madison, WI). LPS purified from *E. coli* K235 was purchased from List Biologicals (Campbell, CA). Contaminating endotoxin proteins were removed by phenol re-extraction as described previously (40, 41). Synthetic lipopeptide corresponding to the N termini of the *Treponema pallidum* 47-kDa lipoprotein (designated 47-L) was a gift from J. Radolf (University of Connecticut, Farmington, CT). Synthetic lipopeptide based on the full-length MALP-2 membrane lipopeptide from *Mycoplasma fermentans* (sMALP-2) was a gift from G. Rawadi (Institut Pasteur, Paris, France) (27, 42). Soluble peptidoglycan (designated sPG) was a gift from R. Dziarski (Indiana University School of Medicine, Gary,

IN). Heat-killed *Listeria monocytogenes* (HKLM) was a gift from G. Teti (Policlinico Universitario, Messina, Italy). Ab to human TLR2, TL2.1, was a gift from T. Espevik (Norwegian University of Science and Technology, Trondheim, Norway). Ab to human TLR4, HTA125, was a gift from K. Miyake (Saga Medical School, Saga, Japan). PE-conjugated sheep anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was used for Ab detection in flow cytometry. In some cases Ab preparations were also directly labeled with Alexa Fluor 488 using a commercially available kit (Molecular Probes, Eugene, OR). A human bone marrow cDNA library was purchased from Invitrogen (Carlsbad, CA).

### Bacteria and bacterial lysates

*N. gonorrhoeae* laboratory strain 1291 was grown overnight on chocolate agar. Colonies were scraped and resuspended in PBS with 0.1% BSA. Cell density was adjusted to an OD<sub>600</sub> equal to 0.1, corresponding to 10<sup>8</sup> CFU/ml bacteria. Crude bacterial lysates were generated from bacteria resuspended in pyrogen-free, sterile distilled water, subjected to a freeze-thaw cycle, and vortexed vigorously. Lysates were treated with DNase for 1 h, stored at -20°C, and vortexed before use.

### Cell lines

Generation of the HPV16/E6E7 immortalized endocervical (End1/E6E7), ectocervical (Ect1/E6E7), and vaginal (Vk2/E6E7) epithelial cell lines has been previously described (8). The vaginal epithelial cells were derived from a different donor than the endocervical and ectocervical cells. Cells were maintained in keratinocyte serum-free medium (Life Technologies, Gaithersburg, MD) supplemented with 50  $\mu$ g/ml bovine pituitary extract and 0.1 ng/ml epidermal growth factor (both supplied by the manufacturer), 10  $\mu$ g/ml ciprofloxacin (Miles Pharmaceuticals, West Haven, CT), and CaCl<sub>2</sub> to a final concentration of 0.4 mM. Cells tested negative for mycoplasma by PCR (43). HMEC-1 (human microvascular endothelial cells) were a gift from M. Ardit (Cedars Sinai Medical Center, Los Angeles, CA) (44). CHO-K1 fibroblasts stably transfected with human TLR2 or TLR4 were a gift from D. Golenbock (University of Massachusetts Medical Center, Worcester, MA) (27). Primary endocervical and ectocervical epithelial cells were purchased from BioWhittaker/Clonetics (San Diego, CA) and maintained according to the manufacturer's instructions.

### RT-PCR analysis

Total RNA was isolated from cells using Tri-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH) and was treated with RQ1 RNase-free DNase (Promega, Madison, WI). Reverse transcription and PCR were conducted using Superscript II (Life Technologies) and Taq DNA polymerase (Promega), respectively. The PCRs were conducted for 25 cycles in a PerkinElmer GeneAmp 2400 PCR machine (PerkinElmer/Cetus, Norwalk, CT) using the primer pairs and conditions described in Table I. Lack of DNA contamination in the RNA preparations was confirmed by PCR in the absence of reverse transcription.

### Detection of soluble mediators

Cells were grown in 96-well tissue culture dishes at a density of 10<sup>5</sup> cells/well and stimulated for the stated period of time. Plates were centrifuged at 400  $\times$  g for 5 min at 4°C before cell culture supernatants were collected. Samples were stored at -80°C and later assayed for IL-8 and IL-6 using PeliKine compact ELISA kits from Research Diagnostics (Flanders, NJ) using the manufacturer's protocol. OD was measured using a Bio-Kinetics microplate reader (Bio-Tek Instruments, Winooski, VT). All cytokine assays were plated in triplicate, and experiments were repeated at least three times. Data are reported as the mean of triplicate samples  $\pm$  SD. Significance (*p* value) was calculated using a *t* test.

### Nuclear extracts and NF- $\kappa$ B translocation assay

One day before stimulation cells were grown in six-well tissue culture dishes at a density of 1  $\times$  10<sup>6</sup>/well. After a 60-min stimulation cells were harvested and nuclear extracts were prepared as previously described (45). Nuclear NF- $\kappa$ B was identified using EMSA with a <sup>32</sup>P-labeled oligonucleotides containing the consensus sequence for NF- $\kappa$ B binding from the murine Ig  $\kappa$  L chain gene enhancer (45).

### Expression plasmids

The following expression plasmids were gifts from D. Golenbock (University of Massachusetts Medical Center): reporter plasmid pELAM-luc, which transcribes firefly luciferase from an NF- $\kappa$ B-dependent promoter (16); mutant murine MyD88 dominant-negative (MyD88 DN), containing the C-terminal half (aa 146–296) of the protein in the mammalian expression vector pLEP3 (46); and mutant murine TLR2 dominant-negative,

Table I. PCR primer pairs for amplification of human mRNA

Product	Upper Primer	Lower Primer	Program	Product Size (bp)
TLR-1	ACCAAGTTGTGTCAGCGATGTGTT	GATTGTCCCCTGCTTTTATTGA	95°C/30 s, 54°C/45 s, 72°C/45 s	660
TLR-2	GAGTGAGTGGTGCAAGTATGAAC	GGGCCACTCCAGGTAGGTCT	95°C/30 s, 54°C/45 s, 72°C/45 s	169
TLR-3	TCCCAAGCCTTCAACGACTG	TCCTGAAAGCTGGCCCGAAAAC	95°C/30 s, 55°C/45 s, 72°C/45 s	471
TLR-4	TGCGGGTTCTACATCAAA	CCATCCGAATTATAAGAAAAGTC	95°C/30 s, 50°C/45 s, 72°C/45 s	413
TLR-5	CTCCTTTGATGGCCGAATAGC	CCCAAATGAAGGATGAAGGTAAAG	95°C/30 s, 55°C/45 s, 72°C/45 s	430
TLR-6	CAAGGCCCTGCCATCTGTAAG	TTGGCCAAAGAAATTGAAAGACTC	95°C/30 s, 54°C/45 s, 72°C/45 s	429
MD-2	GAAGCTCAGAAGCAGTATTGGGTC	GGTTGGTGTAGGATGACAAACTCC	95°C/30 s, 52°C/45 s, 72°C/45 s	422
CD14	GGAACTGACGCTCGAGGACCTAAAGATAAC	TCCAGCCCAGCGAACGACAGATTGAG	95°C/30 s, 60°C/45 s, 72°C/45 s	510
GAPDH	GTTCATCATCTCCGCCCTTCTGTC	GATGCCTGCTTACCACCTTCTTG	95°C/30 s, 54°C/45 s, 72°C/45 s	443

which lacks the terminal 13 aa of the TLR2-coding sequence, expressed in the mammalian expression vector pcDNA3 (46). The expression plasmid for nontagged human TLR4 in the mammalian expression plasmid pcDNA3 was a gift from R. Medzhitov (Yale University, New Haven, CT) (12). The expression plasmid for human MD2 in the mammalian expression plasmid pEFBOS was a gift from K. Miyake (Saga Medical School) (20). All plasmids were prepared using EndoFree Plasmid Maxi Kit plasmid DNA purification columns from Qiagen (Valencia, CA).

#### Transient transfection and NF- $\kappa$ B luciferase reporter assay

Transient transfections were conducted using either DEAE-dextran or a lipid transfection reagent. For the DEAE-dextran method, cells were plated at a density of  $2.5 \times 10^5$  cells/well and transfected with 0.25  $\mu$ g DNA using DEAE-dextran (47). For the lipid transfection, cells were plated at a density of  $10^4$ /well and transfected with 0.1  $\mu$ g DNA using Effectene (Qiagen). Cell activation was determined by measuring luciferase activity in the total cellular lysate using an assay kit from Promega according to the manufacturer's instructions and a Monolight 3010 luminometer (BD PharMingen, San Diego, CA). All transfection experiments were performed in triplicate and repeated at least three times. Data are reported as the mean of triplicate samples  $\pm$  SD. Significance ( $p$  value) was calculated using a  $t$  test.

#### Flow cytometric analysis

Cells growing in tissue culture were harvested with 1 mM EDTA and incubated for 30 min on ice with Alexa-conjugated anti-TLR2 or anti-TLR4 Ab or with control mouse IgG at a concentration of 20  $\mu$ g/ml. After labeling, the cells were washed, resuspended in PBS/1% FBS, and analyzed by flow cytometry using a FACScan microfluorometer (BD Biosciences, San Jose, CA). A total of 10,000 events were counted for each condition.

#### sCD14 assay

Seminal fluid samples were a gift from A. Sunde (Trondheim, Norway). Normal semen was collected at the Department of Obstetrics and Gynecology of the Norwegian University of Science and Technology and processed as previously described (48). Cervical secretions were a gift from P. Crowley-Nowick (Brigham and Women's Hospital, Boston, MA). Samples were collected using the Weck-cel collection method (49) during routine follow-up for abnormal Pap smears at Louisiana State Medical School (Shreveport, LA). Sponges were weighed before and after collection to correct for dilution. sCD14 was assayed by ELISA as described previously (50) (lower limit of detection, 0.78 ng/ml). All samples were taken after obtaining informed consent.

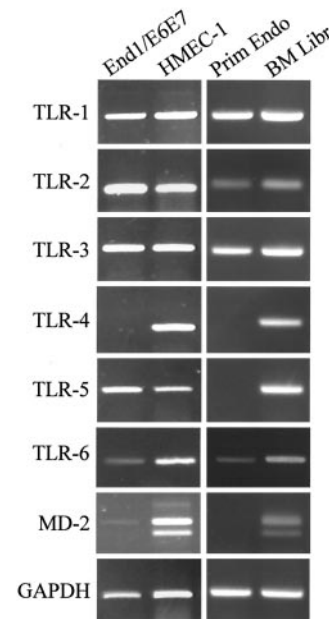
## Results

### Human cervical and vaginal epithelial cells express a distinct repertoire of TLR family members

The immortalized End1/E6E7 epithelial cells and the primary endocervical epithelial cells were evaluated for expression of mRNA for the various TLRs by RT-PCR. HMEC cDNA and a bone marrow cDNA library were used as controls. PCR primers and primer conditions are listed in Table I. While both End1/E6E7 and primary endocervical cells expressed mRNA for TLR1, -2, -3, and -6, no PCR product was detected for TLR4 in either cell population (Fig. 1). The primary cells also failed to express message for

TLR5. In contrast, a TLR4 product of appropriate size was detected in HMEC endothelial cells and the bone marrow library. The endocervical epithelial cells also failed to express a significant amount of message for the TLR4-associated molecule MD2 (Fig. 1). As expected, neither the epithelial cell lines nor the HMEC cells expressed mRNA by RT-PCR for the macrophage differentiation marker CD14 (data not shown). Ectocervical (Ect1/E6E7) and vaginal (Vk2/E6E7) epithelial cells demonstrated a pattern of TLR expression similar to that of the endocervical epithelial cells (data not shown), suggesting that this pattern of expression is consistent throughout the three compartments of the lower female genital tract.

To confirm the PCR finding that the cervical epithelial cells lacked TLR4, we looked for evidence of protein expression. The immortalized End1/E6E7 and primary endocervical epithelial cells were stained using available mAbs to TLR2 (TL2.1) and TLR4



**FIGURE 1.** Endocervical epithelial cells fail to express mRNA for TLR4 and MD2. RNA was prepared from immortalized endocervical epithelial cells (End1/E6E7, left column) and primary endocervical epithelial cells (Prim Endo, right column) and assayed by RT-PCR for expression of TLRs 1–6, MD2, and the housekeeping gene GAPDH as described in the text and Table I. HMEC-1 cDNA and a bone marrow cDNA library (BM Libr) are shown for comparison. A second set of TLR4 primers also failed to yield a product in the epithelial cells. Neither End1/E6E7 epithelial cells nor primary epithelial cells expressed message for CD14 by RT-PCR (data not shown).

(HTA125) and were analyzed by flow cytometry. We found that both the End1/E6E7 cells (Fig. 2A) and the primary endocervical epithelial cells (Fig. 2B) expressed low levels of TLR2 on the surface, but neither expressed TLR4. The specificity of the Abs for TLR2 and TLR4 is demonstrated in Fig. 2C. Of interest, we found that the HMEC endothelial cells expressed surface TLR4 but failed to express TLR2 (data not shown), as has been reported by others (51).

#### Infection of endocervical epithelial cells with *N. gonorrhoeae* activates NF- $\kappa$ B-mediated proinflammatory pathways

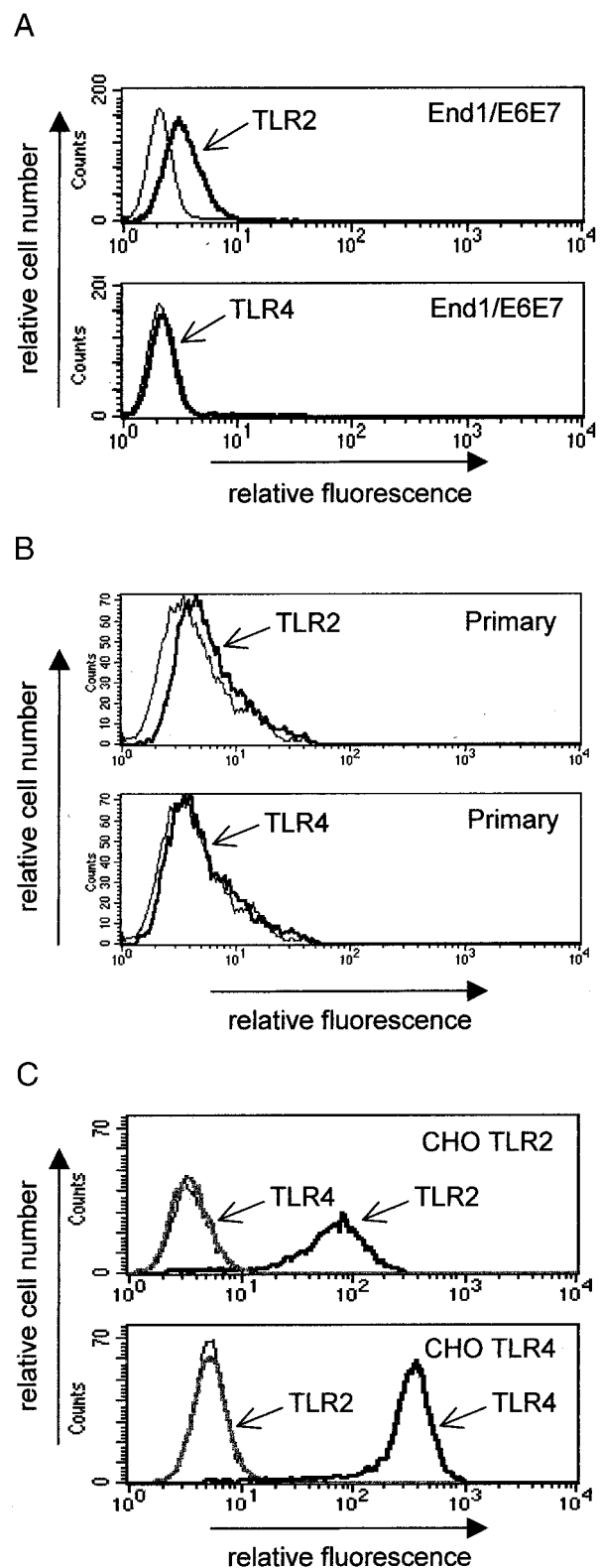
To determine whether *N. gonorrhoeae* infection of epithelial cells could initiate a proinflammatory signal, cells were assayed for a pretranscriptional event, the nuclear translocation of the transcription factor NF- $\kappa$ B. NF- $\kappa$ B activation is central to the inflammatory response to bacterial pathogens, leading to the release of several proinflammatory cytokines and chemokines (reviewed in Ref. 52). We found evidence of NF- $\kappa$ B activation by the EMSA within 1 h of stimulation using both live *N. gonorrhoeae* and bacterial lysates, suggesting that attachment and invasion were not necessary for cellular activation (Fig. 3A). In fact, the bacterial lysates appeared to be more potent than the live bacteria, perhaps reflecting the availability of more inflammatory ligands in the particulates compared with intact bacteria.

During mucosal infections, epithelial cells are a major source of IL-8 and IL-6, which, in turn, participate in local inflammation, neutrophil chemotaxis, T and B cell activation, and Ab production (reviewed in Ref. 53). To investigate a model of natural infection in the endocervical canal, endocervical epithelial cells were incubated with increasing concentrations of *N. gonorrhoeae* lysates and assessed over time for the release of IL-8 and IL-6 into the supernatant. As shown in Fig. 3B, IL-8 concentrations were increased in culture supernatants in a time- and dose-dependent manner when stimulated with bacterial lysates. Similar results were found when supernatants were assayed for IL-6 (data not shown).

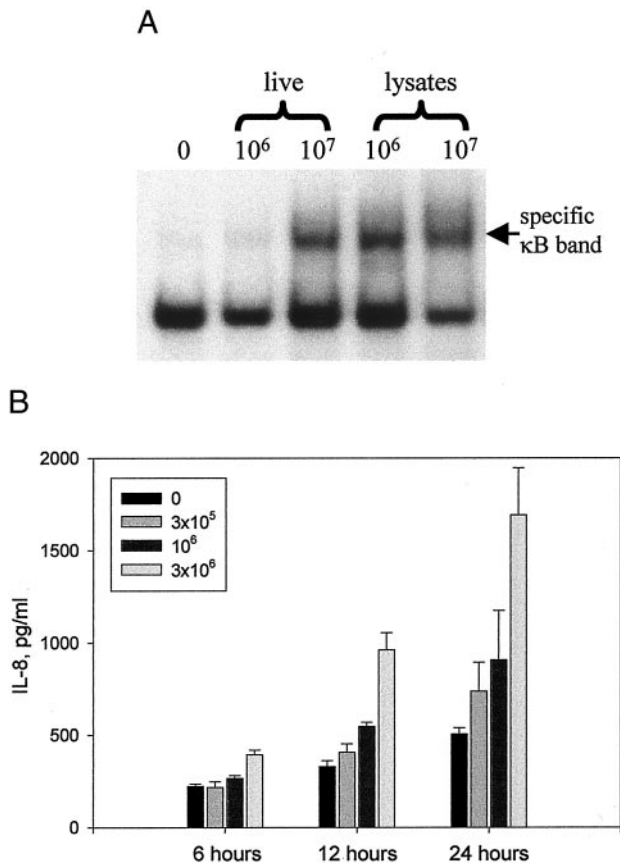
#### sCD14 plays a role in host epithelial cell responses to *N. gonorrhoeae* in the lower female genital tract

Because the cells are grown in serum-free medium, they are not exposed to the serum proteins sCD14 (54–56) and LBP (57), which have been shown by many groups to enhance responses to LPS. sCD14 has also been shown to interact with other bacterial ligands, including mycobacterial lipoarabinomannan (58) and mycobacterial cell wall components (50), peptidoglycan (59, 60) and cell wall components of *Staphylococcus aureus* (61), and bacterial outer membrane lipoproteins (62). When the culture medium was supplemented with sCD14, the endocervical epithelial cells were more sensitive to activation by *N. gonorrhoeae* lysates, with the cytokine response to stimulatory concentrations of bacteria enhanced at all levels of sCD14 tested (Fig. 4). Furthermore, high levels of sCD14 (>100 ng/ml) enabled cellular responses to normally substimulatory concentrations of bacteria (Fig. 4). Identical results were obtained using live bacteria (data not shown). No effect of LBP, either by itself or in combination with sCD14, was seen (data not shown).

To confirm a physiologic role for sCD14 in genital tract infections, we looked for the presence of this molecule in cervical mucus of women and in semen of men. Of 10 samples of mucosal secretions collected from the cervix, only one had sCD14 detectable at a concentration of 194.2 ng/ml. However, all cervical samples were diluted in the range of 10- to 40-fold during the collection process, which could account for the inability to detect relatively low levels of the protein (<10–20 ng/ml) that might still be physiologically relevant under some conditions. In contrast,



**FIGURE 2.** Endocervical epithelial cells express surface TLR2, but not TLR4. Shown above are FACS histograms for the End1/E6E7 cell line (A), primary endocervical epithelial cells (B), and TLR2- or TLR4-transfected CHO-K1 fibroblasts (C). Cells were stained with Ab to TLR2 (TL2.1, upper graph) or TLR4 (HTA125, lower graph) as described in the text. In each case the staining is compared with that of control mouse IgG (thin solid line). The vertical axis represents the relative cell number, while the horizontal axis represents the intensity of fluorescence staining for the Ab. These histograms are representative of at least three separate experiments.



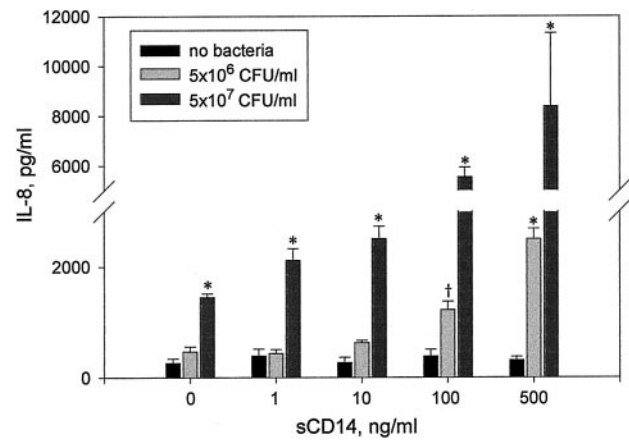
**FIGURE 3.** *N. gonorrhoeae* activates proinflammatory signals in endocervical epithelial cells. **A**, NF- $\kappa$ B translocation. End1/E6E7 cells were stimulated with live *N. gonorrhoeae* or gonococcal lysates for 60 min. Nuclear proteins were prepared, and nuclear levels of NF- $\kappa$ B were measured by EMSA using a  $\kappa$ B site-containing probe. The upper band represents NF- $\kappa$ B bound to the  $\kappa$ B. Shown above is a representative of at least three independent experiments. **B**, IL-8 release. End1/E6E7 cells were stimulated with increasing concentrations of *N. gonorrhoeae* lysates, and supernatant was harvested over time and assayed for IL-8 by ELISA. Shown above is a representative of at least three independent experiments. Similar results were found when supernatant was assayed for IL-6 (data not shown).

seminal plasma contained abundant sCD14, with levels approximating half those in serum (Fig. 5).

*Endocervical epithelial cells are unresponsive to LPS, but maintain proinflammatory responses to other cell wall components of Gram-negative bacteria*

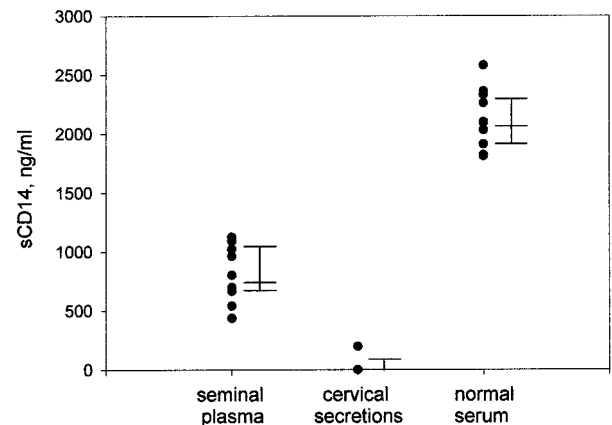
Various components of the bacterial cell wall are capable of activating the proinflammatory response. In the case of Gram-negative bacteria, LPS, or more specifically the lipid A core of LPS, is believed to be the molecule responsible for the biological toxicity (63). However, other components of the Gram-negative cell wall, such as proteins, lipoproteins, peptidoglycan, porins, and a variety of phospholipids, have been shown in other settings to activate of the inflammatory response. Furthermore, an LPS-deficient mutant of *Neisseria meningitidis* has been shown to be a potent inflammatory stimulus (64).

Because endocervical epithelial cells lacked the known components of the LPS signaling complex, specifically TLR4 and MD2, we suspected that they would be unresponsive to LPS. To test this, cells were incubated with increasing amounts of LPS and other purified bacterial products and assessed for IL-8 and IL-6 release.

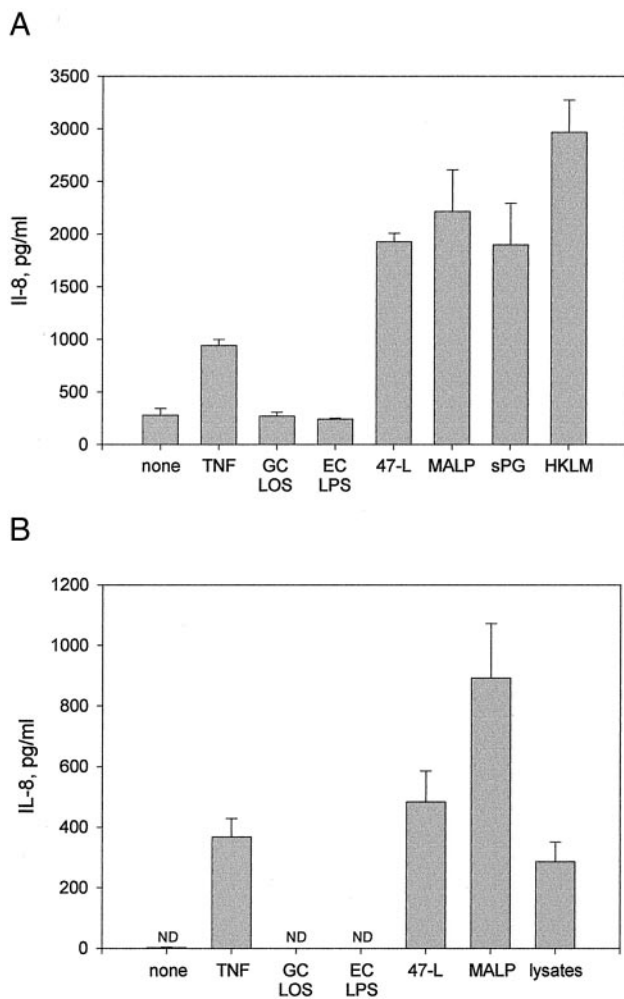


**FIGURE 4.** Cellular activation by *N. gonorrhoeae* is enhanced by sCD14. End1/E6E7 cells were stimulated with *N. gonorrhoeae* lysates in the presence of increasing concentrations of sCD14. Supernatant was collected after 18 h and assayed for IL-8 by ELISA. Shown is a representative of at least three independent experiments. Similar results were found when supernatant was assayed for IL-6 (data not shown). Significance is noted as follows: \*,  $p < 0.0001$ ; †,  $p < 0.001$  (stimulated vs unstimulated).

Considering the requirement of sCD14 for LPS responses in cells lacking membrane CD14, medium was supplemented with recombinant sCD14 (54–56). As demonstrated in Fig. 6A, the immortalized endocervical epithelial cells failed to increase IL-8 secretion in response to protein-free preparations of endotoxin derived from *N. gonorrhoeae* and *E. coli*. They also failed to translocate the transcription factor NF- $\kappa$ B as assayed by the EMSA upon LPS stimulation (data not shown). In contrast, they responded quite well to other bacterial preparations, including synthetic bacterial lipopeptides from *T. pallidum* (47-L) and *M. fermentans* (sMALP-2), sPG, and HKLM. All these preparations have been shown previously to be ligands for TLR2 (23–27, 29), or TLR2 in concert with TLR6 (30). Likewise, primary endocervical epithelial cells were activated by gonococcal lysates and the TLR2 ligands 47-L and sMALP-2, but were unresponsive to the two LPS preparations tested (Fig. 6B). Primary ectocervical epithelial cells were also LPS unresponsive (data not shown).



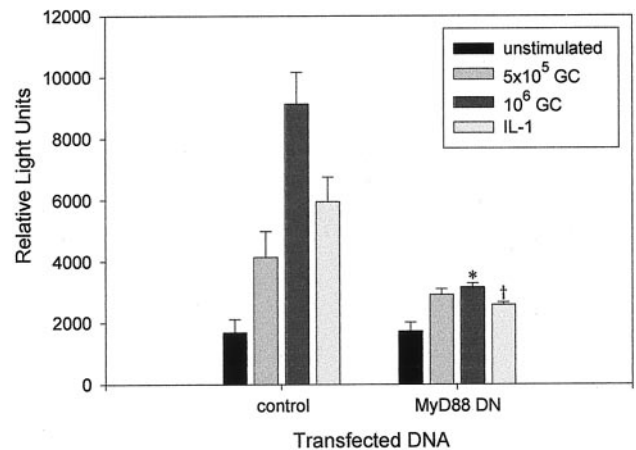
**FIGURE 5.** sCD14 is detectable in seminal plasma. Samples of seminal plasma and cervical secretions ( $n = 10$  for each) were assayed for sCD14 by ELISA as described in *Materials and Methods*. Levels of sCD14 in normal serum ( $n = 11$ ) are shown for comparison. The bars indicate the medians (middle line) and 25th (upper line) and 75th (lower line) percentiles.



**FIGURE 6.** Endocervical epithelial cells are unresponsive to LPS. End1/E6E7 (A) and primary endocervical (B) cells were plated in serum-free medium supplemented with 200 ng/ml sCD14 and stimulated overnight with the following: 4 ng/ml human TNF- $\alpha$ , 1  $\mu$ g/ml *N. gonorrhoeae* LOS (GC LOS) or *E. coli* LPS (EC LPS), 5  $\mu$ g/ml synthetic lipopeptide 47-L, 20 nM synthetic lipopeptide sMALP-2, 5  $\mu$ g/ml sPG, or  $10^8$  CFU/ml HKLM. Supernatant was collected after 18 h and assayed for IL-8. The results represent at least three independent experiments. ND, None detected. Results were confirmed in primary endocervical epithelial cells from a second donor. Primary ectocervical epithelial cells were also unresponsive to LPS (data not shown).

#### Endocervical epithelial cells use the Toll/IL-1 pathway

The C-terminal domain of MyD88 has been shown to act as a dominant-negative inhibitor of NF- $\kappa$ B activation by both IL-1 (65) and many of the TLRs (46). We hypothesized that such a MyD88-DN mutant would block the *N. gonorrhoeae*-induced NF- $\kappa$ B activation that we observed in our epithelial cells. To test this idea, we used an NF- $\kappa$ B-dependent ELAM-luciferase reporter. Cells were transiently cotransfected with the reporter plasmid and either the expression plasmid containing the dominant-negative MyD88 construct or a control vector. Cells were then stimulated with increasing concentrations of the gonococcal lysates and assayed for activation of the luciferase reporter. As shown in Fig. 7, overexpression of the dominant-negative MyD88 blocked gonococcal-induced activation of the luciferase reporter as well as that of IL-1 $\beta$ , which uses the same intracellular signals as the TLRs. In contrast to MyD88, we were unable to see consistent inhibition of the crude lysates with either a TLR2 dominant-negative construct



**FIGURE 7.** TLR proteins mediate the activation of endocervical epithelial cells by *N. gonorrhoeae*. End1/E6E7 cells were transiently cotransfected with an expression plasmid encoding a dominant-negative MyD88 mutant or empty vector and with a luciferase reporter plasmid. Cells were then stimulated with increasing concentrations of gonococcal lysates or IL-1 and assayed for luciferase activity as described in the text. Shown above is a representative of at least three independent experiments. Significance is noted as follows: \*,  $p = 0.0001$ ; †,  $p = 0.001$  (MyD88DN vs control).

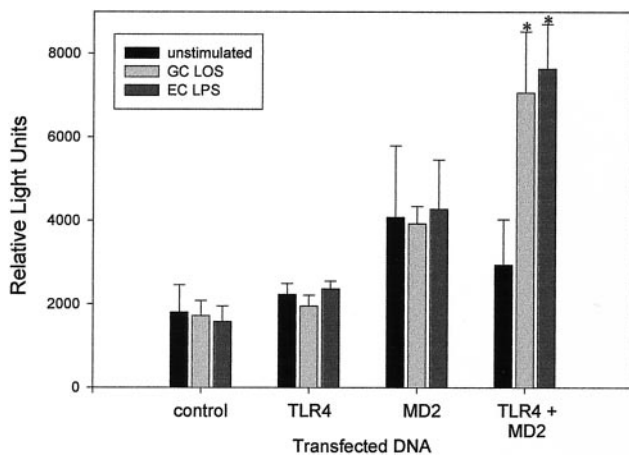
or TLR2-blocking Abs, suggesting that the ligands in the gonococcal lysates responsible for the cellular activation were using more than one TLR in the MyD88-dependent activation (data not shown).

#### Transfection of TLR4 and MD2 restores LPS responsiveness in the endocervical epithelial cell line

We hypothesized that the absence of TLR4 and MD2 was responsible for the LPS unresponsiveness by epithelial cells in the lower female genital tract. To test this hypothesis, we transiently cotransfected the endocervical epithelial cell line with the ELAM-luciferase reporter plasmid and the expression plasmids containing the genes for human TLR4 and MD2. Cells were then stimulated with both *N. gonorrhoeae* LOS and *E. coli* LPS, in the presence of sCD14 and assayed for activation of the luciferase reporter. As predicted, we found that the combination of TLR4 and MD2 conferred responsiveness to the two different LPS preparations, while expression of TLR4 or MD2 alone did not (Fig. 8).

## Discussion

The epithelium of the lower female genital tract is a dynamic tissue, changing under the influence of hormones, age, pregnancy, and other factors. While the stratified squamous epithelia of the human vagina and ectocervix are exposed to the complex microenvironment of the vagina, the columnar epithelium of the endocervix is generally considered a sterile site, in part due to the cervical mucus that filters bacteria and other debris while allowing sperm to ascend to the upper genital tract. However, this barrier can readily be crossed by a variety of infectious agents, in particular *N. gonorrhoeae* and *Chlamydia trachomatis*, typically leading to cervicitis. The lower female genital tract can also become contaminated with Gram-negative organisms secondary to the presence of rectal flora in the perineal area. Thus, it is essential that the normal cervical epithelium have the capacity to recognize and respond to ascending pathogens while at the same time avoiding a



**FIGURE 8.** Expression of TLR4 and MD2 together restores LPS responsiveness. End1/E6E7 cells were transiently cotransfected with an expression plasmid encoding TLR4, MD2, both, or an empty vector, along with the luciferase reporter plasmid. Cells were then stimulated with 1  $\mu$ g/ml endotoxin prepared from either *N. gonorrhoeae* (GC LOS) or *E. coli* (EC LPS). Lysates were assayed for luciferase activity as described in the text. Shown above is a representative of two independent experiments. Significance is noted as follows: \*,  $p = 0.001$  for LOS and LPS vs unstimulated.

state of chronic inflammation that might disrupt the epithelial barrier. The sequelae of such chronic inflammation in the female genital tract would be highly detrimental to the host and include increased transmission of sexually transmitted diseases, especially HIV (7), and increased risk of preterm delivery (66).

Our results demonstrate that epithelial cells in the lower genital tract have evolved a unique mechanism for the recognition of Gram-negative pathogens. In contrast to professional phagocytic cells, the epithelial cells of the mucosal surface lack the key components of the LPS response machinery, TLR4 and MD2, and, consistent with this, lack cytokine responsiveness to purified LPS preparations. Epithelial cells in other mucosal sites have also been shown to have various degrees of LPS responsiveness. Perhaps most confusing are the data on gastrointestinal epithelial cells, where the ability of LPS to up-regulate proinflammatory cytokines appears to vary with the cell line tested (67–70). In addition, airway epithelial cells have demonstrated sensitivity to LPS by NF- $\kappa$ B activation, cytokine production, and up-regulation of various defense molecules (71–74), and LPS has been shown to stimulate cytokine expression by human gingival epithelial cells (75–77). None of these studies identified host cell surface moieties linked to epithelial-LPS interactions.

Although considerable effort has been aimed at the elucidation of TLR signaling in professional immune cells, the roles of various TLR-related molecules in mucosal epithelial cell recognition of bacterial components remain poorly understood, with gastrointestinal epithelial cells having been studied the most extensively. For example, colon cancer cell lines as well as intestinal epithelial cells derived from patients with idiopathic inflammatory bowel disease have been reported to express functional TLR4 (78). In contrast, epithelial cells in normal adult intestinal tissue appear to be TLR4 deficient by immunohistochemistry (79), and it has been reported that several intestinal epithelial cell lines lack TLR4 and MD2, accounting for their LPS hyporesponsiveness (80). In addition, Asai et al. (81) recently reported that gingival epithelial cells also fail to express TLR4 and respond to Gram-negative bacteria via TLR2 ligands.

Our findings are the first to characterize the expression of the known TLRs in normal, nontumorigenic epithelial cells and the first to examine the expression in the mucosal surface of the female genital tract. The similarity between our data and the recent reports on digestive tract epithelium by Abreu et al. (80) and Asai et al. (81) suggests that nonsterile mucosa has evolved a unique mechanism for bacterial recognition that differs from that used in sterile sites. The unique TLR expression pattern, in particular the lack of TLR4 and the associated molecule MD2, suggests that the cervicovaginal epithelium is capable of responding to Gram-negative pathogens in the absence of endotoxin recognition. This is in contrast to the mechanism used by phagocytes (15, 17, 82), dendritic cells (83), and endothelial cells (51, 84), which express TLR4 and are highly sensitive to LPS activation through the Toll/IL-1 signaling pathway.

We can only speculate about the possible evolutionary pressure that would lead to such differential expression of TLR4. It has been suggested that the lack of TLR4 expression in intestinal epithelial cells might prevent the constant proinflammatory gene activation that could occur with exposure to normal enteric flora and might account for some of the chronic intestinal inflammation associated with disorders such as inflammatory bowel disease (78–80). The lower female genital tract also has a complex ecosystem, and it is possible that a similar model might exist in this compartment as well. Certainly, a threshold of sensitivity to bacterial components in the genital tract is required to avoid unnecessary inflammation. While inflammation is essential for clearing bacterial infections, excessive inflammation is particularly detrimental to the defense function of the mucosal surface. For example, it has been shown that inflammation increases the risk of sexual transmission of pathogens such as HIV-1 (7).

Our results also suggest that sCD14 has an LPS/TLR4-independent role in the genital tract epithelial cell responses to Gram-negative bacteria, enhancing the proinflammatory cytokine response to live bacteria and bacterial lysates. Even low concentrations of sCD14 (1–10 ng/ml) that would be below the detection limit of our assay because of sample dilution have effects on the cytokine response when the inoculum of bacteria is high. Furthermore, high concentrations of sCD14, as might be found during menses or following intercourse, may act to fortify the host defense under conditions of increased risk of infection by augmenting the inflammatory cytokine response. The precise molecular mechanism by which sCD14 enhances TLR-mediated cellular responses to bacterial membranes is unclear. Others have shown that membrane CD14 can facilitate signaling via TLR2 ligands including lipoproteins (62, 85) and peptidoglycan (23, 24). An enhancing effect of sCD14 was also found by Asai and colleagues regarding *Porphyromonas gingivalis* fimbrial lipoproteins and TLR2 (81). Our data go further to suggest that sCD14 is capable of enhancing the proinflammatory response not only to purified ligand, but also to bacterial lysates and live bacteria. This could be accomplished either by directly presenting ligand to a TLR or by increasing the availability of ligands by interactions with the bacterial membrane.

Thus, while TLR4 may be essential for the response to Gram-negative pathogens during bacteremia and sepsis, other TLRs may be more important for the recognition of Gram-negative bacteria at specific sites, such as the mucosal surface of the female genital tract. A better understanding of the regulation of TLR expression and its influence on bacterial recognition may provide insight into the pathogenesis of a variety of infectious diseases, in particular the pathophysiology of sexually transmitted diseases and the deleterious sequelae associated with chronic genital tract inflammation.



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