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CCL20/Macrophage Inflammatory Protein 3α and Tumor Necrosis Factor Alpha Production by Primary Uterine Epithelial Cells in Response to Treatment with Lipopolysaccharide or Pam_3Cys

Mardi A. Crane-Godreau* and Charles R. Wira

Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire

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Having previously shown that CCL20/macrophage inflammatory protein 3α and tumor necrosis factor alpha (TNF-α) are released by polarized primary rat uterine epithelial cells (UEC) in response to *Escherichia coli* but not to *Lactobacillus rhamnosus*, we sought to determine if epithelial cells are responsive to pathogen-associated molecular patterns (PAMP), including lipopolysaccharide (LPS), lipoteichoic acid (LTA), and Pam_3Cys, a bacterial lipoprotein analog. Epithelial cells were grown to confluence on Nunc cell culture inserts prior to apical treatment with PAMPs. In response to LPS, LTA, and Pam_3Cys (EMC Microcollection GmbH, Tübingen, Germany), CCL20 levels increased (4- to 10-fold) while PAMPs caused increased TNF-α (1- to 4-fold) in the medium collected after 24 h of incubation. Both apical and basolateral secretion of CCL20 and TNF-α increased in response to PAMPs, but treatments had no effect on cell viability and integrity, as measured by transepithelial resistance. Time course studies of CCL20 and TNF-α release in response to Pam_3Cys and LPS indicated that CCL20 release peaked between 2 and 4 h after treatment, whereas TNF-α release was gradual over the length of the incubation. Freeze-thaw and cell lysis experiments, along with actinomycin D studies, suggested that CCL20 and TNF-α are synthesized in response to PAMP stimulation. Taken together, these studies demonstrate that *E. coli* and selected PAMPs have direct effects on the production of CCL20 and TNF-α without affecting cell integrity. Since CCL20 is known to be both chemotactic and antimicrobial, the increase in apical and basolateral release by UEC in response to PAMPs suggests a new mechanism of innate immune protection in the female reproductive tract.

Epithelial cells are the host's first line of defense against potential pathogenic microbes (6, 11). At mucosal surfaces, including those of the female reproductive tract, epithelial cells have evolved as part of the innate immune system, with antimicrobial functions as well as the ability to influence the presence and activity of immune cells. The innate immune system differs from the adaptive immune system in the types and specificities of receptors for antigens, the immediacy of the response, and the cell types involved (24, 37). Innate immune protection relies on conserved germ line-encoded receptors and molecules that recognize conserved pathogen-associated molecular patterns (PAMPs) found in groups of microorganisms. PAMPs are recognized by Toll-like receptors (TLRs) that are expressed on many cell types, including macrophages (22), dendritic cells (25), and epithelial cells (4). Lipoteichoic acid (LTA), a bacterial cell wall component derived from gram-positive bacteria, and Pam_3Cys, a synthetic analog of bacterial lipopeptides, bind TLR2/1 heterodimers. Lipopolysaccharide (LPS), from gram-negative bacteria, binds TLR4 homodimers (19–21).

Cells of the innate immune system regulate immune responses through the production of chemokines and cytokines, including CCL20, transforming growth factor beta (TGF-β), and tumor necrosis factor alpha (TNF-α), that recruit and activate immune cells at mucosal surfaces throughout the body, including the female reproductive tract (10, 28, 42). CCL20, which is chemotactic for bone marrow-derived dendritic cell precursors, B cells, and memory T cells which express the CCR6 receptor, is released by rat uterine epithelial cells (UEC) and the human uterine epithelial cell line HHUA (8, 35, 52). CCL20 production has been studied in several other tissues, including epithelial cells of the airways and the gastrointestinal tract (31, 34, 43). Beyond its role as a chemokine, CCL20 is structurally and functionally similar to the family of antimicrobial molecules known as β-defensins, which share an affinity for the CCR6 receptor (23, 48). TGF-β is released by polarized uterine epithelial cells in culture and can act as a cytokine that can influence chemotaxis (8, 17, 18, 47, 58). This molecule regulates uterine epithelial cell proliferation and apoptosis (46) and has recently been shown to influence antigen presentation by uterine and vaginal cells (56, 58). TNF-α plays a role in the acute response to infection and in apoptosis (26, 29). The fact that these cytokines are interconnected is exemplified by the ability of TNF-α to upregulate CCL20 transcription (51). In other studies, TGF-β downregulated the production of TNF-α (50). Both TGF-β and TNF-α have been shown to play a role in regulating dendritic cell maturation and cell trafficking (5, 17)

Previously, we and others have shown that uterine epithelial cells are actively involved in immune protection through the expression of polymeric immunoglobulin receptors that transport immunoglobulin A (IgA) from the uterine tissue to the lumen (12, 45) as well as through the release into the lumen of antimicrobial molecules, such as secretory leukocyte protease inhibitor (SLPI), that are bactericidal to both gram-negative

* Corresponding author. Mailing address: Department of Physiology, Dartmouth Medical School, Borwell Building, 1 Medical Center Dr., Lebanon, NH 03756. Phone: (603) 650-7733. Fax: (603) 650-6130. E-mail: Mardi.Crane@Dartmouth.edu.

and gram-positive bacteria (14, 54). In other studies, uterine epithelial cells have been shown to present antigens through class II molecules to T cells as part of the onset of adaptive immunity (13, 57). More recently, we found that uterine epithelial cells in culture respond to the presence of live and heat-killed *Escherichia coli* by releasing increased amounts of CCL20 and TNF-α (8). In contrast, TGF-β release was inhibited by live *E. coli*. Unexpectedly, we found that live and heat-killed *Lactobacillus rhamnosus*, a known commensal found in the female reproductive tract (44), had no effect on epithelial cell signaling.

The overall goal of the present study was to more fully define the mechanism(s) through which uterine epithelial cells respond to a bacterial challenge. We tested the hypothesis that primary rat uterine epithelial cells respond to bacterial cell wall components, specifically LPS, LTA, and Pam_3Cys, by releasing CCL20, TNF-α, and TGF-β. Our objectives were to (i) establish whether selected PAMPs affect the release of CCL20, TNF-α, and TGF-β by rat epithelial cells, (ii) identify the pattern of release of CCL20 and TNF-α in response to PAMP exposure, and (iii) determine if CCL20 and TNF-α are produced de novo in response to PAMP stimulation.

MATERIALS AND METHODS

General procedures. Uterine epithelial cells were harvested from specific-pathogen-free Lewis rats weighing from 125 to 175 g (Charles River Breeding Laboratories, Kingston, N.Y.). The rats were maintained with alternating 12-h dark-light cycles and were given free access to food and water. All procedures involving animals were conducted according to protocols approved by the Dartmouth College Institutional Animal Care and Use Committee.

Preparation of epithelial cell cultures. All epithelial cell cultures described here were primary cultures that were established by pooling the uteri of six or more randomly selected animals at various stages of the estrous cycle. Rat uteri were removed, rinsed in sterile ice-cold Hanks' balanced salt solution (Gibco, Grand Island, N.Y.), weighed, and then digested with pancreatin (Gibco), trypsin (Sigma, St. Louis, Mo.), and DNase (Worthington, Lakewood, N.J.) (400 U of DNase/ml of pancreatin, 46,500 U of trypsin/ml of pancreatin, 19.5 ml of pancreatin/g of uterine tissue) as previously described (8). The uteri were cut into fine pieces and incubated for 1 h at 4°C, followed by an additional hour at room temperature. The tissues were vortexed prior to passage through a sterile 250-μm-pore-size mesh screen. Epithelial cell sheets were recovered by pouring the resulting suspension onto a 20-μm-pore-size mesh capture screen. After being rinsed to remove stromal contamination, UEC sheets were then collected, suspended in F12K medium (American Type Culture Collection, Rockville, Md.) plus 10% fetal bovine serum supplemented with 100 μg of streptomycin/ml and 100 U of penicillin/ml (complete F12K medium), and plated on growth factor-reduced Matrigel-coated Nunc 10-mm by 0.4-μm polycarbonate membrane inserts (Nalgene Nunc International) at a density of three to four cell culture insert wells per rat uterus. Characterizations of epithelial cell cultures and preparation were previously reported (45, 57).

Monitoring development of polarized epithelial cell cultures. Uterine epithelial cells were incubated in complete F12K medium at 37°C in 5% CO_2 throughout the experimental period. Using established methods, we monitored the development and polarization of viable UEC monolayer cultures by measuring transepithelial resistance (TER) (16) on an EVOM voltohmmeter (World Precision Instruments). UEC were incubated with PAMPs only after a high TER (1,000 to 3,500 Ω/well), consistent with a confluent epithelial cell monolayer devoid of stromal contamination, was established.

Heat-killed bacterial preparations. *L. rhamnosus* (ATCC 7469) and *E. coli* (ATCC 29839) obtained from the American Type Culture Collection and grown in sterile de Man, Rogosa, and Sharp broth and Trypticase soy broth, respectively, were maintained as previously described (8). Bacterial counts were determined by optical density analyses of bacterial saline suspensions, followed by serial dilutions, plating on agar, and triplicate plate counting. For preparations of heat-killed bacteria, *L. rhamnosus* and *E. coli* were grown to stationary phase, suspended in sterile saline, and placed on ice for 2 h. The bacterial suspensions were placed in sterile glass tubes and immersed in a 70°C water bath for 20 min. Heat-killed bacterial suspensions were cooled, vortexed, divided into aliquots, and frozen at −20°C.

Treatment of epithelial cells with heat-killed bacteria and PAMPs. LPS (from *E. coli*) (L4391; Sigma), LTA (from *Bacillus subtilis*) (L3265; Sigma), Pam_3Cys (L2000; EMC Microcollections GmbH, Tübingen, Germany), and repurified (ultrapure) LPS (from *Salmonella minnesota*) (R595; List Biological Laboratories, Campbell, Calif.) were reconstituted under sterile conditions. PAMPs were tested by a *Limulus* assay (*Limulus* amebocyte lysate QCL-1000; Biowhittaker) for the presence of endotoxin. Pam_3Cys and LTA (1 μg/ml) contained <0.018 and 2.6 endotoxin units/ml, respectively. Ultrapure LPS (10 ng/ml) exceeded the range of sensitivity of the assay. Cell cultures were treated with PAMPs in the apical compartment. Fresh medium was placed in the apical and basolateral compartments at the beginning of each treatment period. The basolateral medium was harvested at the end of the experimental period(s) for analysis.

Measurements of CCL20, TNF-α, and TGF-β. The amounts of CCL20 and TNF-α were determined by use of an enzyme-linked immunosorbent assay (ELISA) kit for rat macrophage inflammatory protein 3α/CCL20 or rat TNF-α (DuoSet ELISA development system; R&D Systems, Minneapolis, Minn.). Biologically active TGF-β was measured by a bioassay using a mink lung epithelial (MLE) cell line transfected with the plasminogen activator 1 promoter linked to a luciferase reporter gene as previously described (8). This bioassay is based on the MLE cell line's specific sensitivity to picogram levels of biologically active TGF-β, which includes the expression of plasminogen activator 1 and results in a consistent dose-dependent luciferase activity. Briefly, transfected MLE cells were seeded into a 96-well plate at 10^5/100 μl of medium/well and then were spun in a Beckman centrifuge at 1,500 rpm for 15 s. After an initial 3-h incubation at 37°C, the medium was removed and replaced with 50 μl of fresh medium plus 50 μl of serially diluted standard (recombinant human TGF-β) or culture medium. The cells were cultured for 17 h prior to lysing by the addition of 50 μl of cell culture lysis reagent (Promega)/well for 15 min. Luciferase activities were determined by the responses of MLE cell lysates to 100 μl of luciferase reagent (Promega)/well for 10 s in a microplate luminometer (model LB96V; EG&G Berthold, Gaithersburg, Md.).

Statistics. Data were compared by one-way analysis of variance followed by Tukey's multiple comparison posttest. Differences with P values of <0.05 were considered significant.

RESULTS

Influence of heat-killed *E. coli* and LPS on release of CCL20 and TNF-α by polarized uterine epithelial cells. We previously showed that uterine epithelial cells, when grown to confluence on cell culture inserts, respond to live and heat-killed *E. coli* with increased CCL20 and TNF-α secretion into the basolateral chamber, while they show no change in cytokine release in response to *L. rhamnosus* (8). To more fully understand the nature of these bacterial interactions, we exposed epithelial cell cultures to heat-killed bacteria and compared their levels of responsiveness to LPS, an antigenic component of the gram-negative bacterial cell wall. Polarized rat uterine epithelial cells grown on Nunc cell culture inserts in complete F12K medium were treated apically with 10^6 CFU of heat-killed *E. coli* or *L. rhamnosus* or 10 μg of commercial-grade LPS/ml. After incubation for 24 h, the basolateral medium was harvested and analyzed for CCL20 and TNF-α by ELISA. As shown in Fig. 1, the presence of LPS at the apical surfaces of rat uterine epithelial cells increased the basolateral release of CCL20 and TNF-α, similar to what was seen for cultures treated with 10^6 CFU of heat-killed *E. coli*. As reported previously (8), apical treatment of the UEC cultures with heat-killed *L. rhamnosus* had no effect on the release of either CCL20 or TNF-α. In all cases, irrespective of the bacteria or PAMP used, the TER was not significantly different from that of controls, consistent with the presence of a viable polarized epithelial cell monolayer (data not shown).

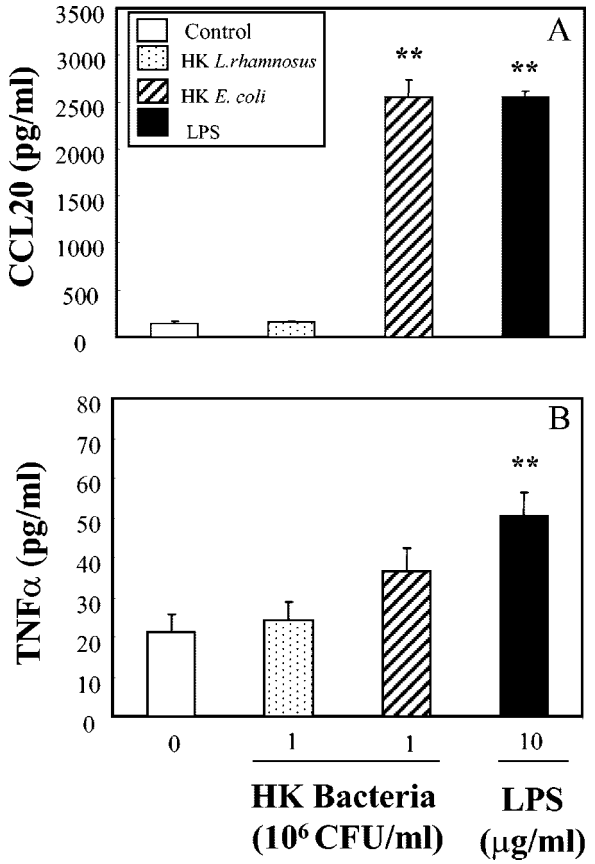

FIG. 1. Effect of heat-killed bacteria and LPS on uterine epithelial cell release of CCL20 (A) and TNF-α (B). Polarized rat uterine epithelial cells grown in F12K complete medium on Nunc cell culture inserts were treated apically with heat-killed bacteria or LPS in complete medium. The basolateral medium was removed after a 24-h incubation for analyses of CCL20/MIP-3α and TNF-α by ELISA. Values are means ± standard errors (SE) for five wells per group. **, significantly ($P < 0.01$) different from control. The data are representative of three separate experiments.

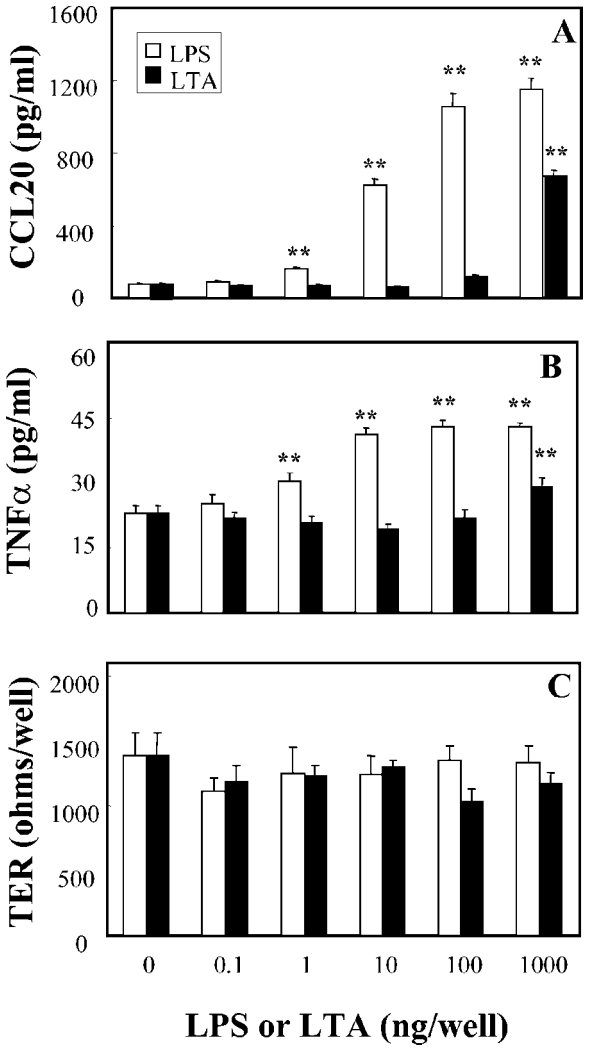

FIG. 2. Dose response of rat uterine epithelial cells to LPS and LTA. Rat uterine epithelial cells cultured on Nunc cell culture inserts in F12K complete medium were treated apically with LPS or LTA in complete medium. The basolateral medium was removed after a 24-h incubation and analyzed by ELISA for CCL20 (A) and TNF-α (B). (C) The TER was measured to determine the presence of a polarized monolayer of UEC. Values are means ± SE for five wells per group. **, significantly ($P < 0.01$) different from control. Data are representative of three separate experiments.

Response of uterine epithelial cells to apical treatment with LPS, Pam_3Cys, and LTA. To more fully characterize the interaction of PAMPs with polarized rat uterine epithelial cells, we measured cytokine release and TER in response to treatment with increasing doses of LPS or LTA isolated from the cell wall components of gram-negative (LPS) and gram-positive (LTA) bacteria. As shown in Fig. 2C, TERs of 1,000 to 1,500 Ω/well were not affected when commercial-grade LPS or LTA was added to the apical compartment for 24 h in doses ranging from 0.1 to 1,000 ng/well. When the basolateral medium from cell cultures exposed to LPS was analyzed by ELISA, CCL20 and TNF-α levels were shown to increase in a dose-dependent manner (Fig. 2A and B). Moreover, we found that LTA administered at 1,000 ng/ml, but not at lower doses, also elicited an increase in CCL20 and TNF-α.

Studies of cellular responses to LPS have shown that repurification of LPS by a phenol extraction of bacterial lipoproteins eliminates signaling through TLR2, suggesting that commercial-grade LPS is contaminated with fragments of other bacterial cell wall components (21). To more fully define the response of UEC to bacterial antigens, we performed a dose-response experiment in which epithelial cells were incubated for 24 h with repurified ultrapure LPS, a pure agonist of TLR4. Figure 3 indicates that ultrapure LPS increases the release of CCL20 by polarized epithelial cells when it is added to the apical compartment in doses ranging from 100 pg to 1 ng/ml. In contrast, significantly more ultrapure LPS (10 to 100 ng/ml) was needed to increase the basolateral release of TNF-α (not shown). In other studies to determine if LPS affects the release of biologically active TGF-β by UEC, we measured TGF-β in the basolateral compartment. In contrast to the effect of live *E. coli*, which decreases the release of TGF-β (8), we found that neither commercial-grade LPS nor repurified LPS had any effect on TGF-β release (data not shown).

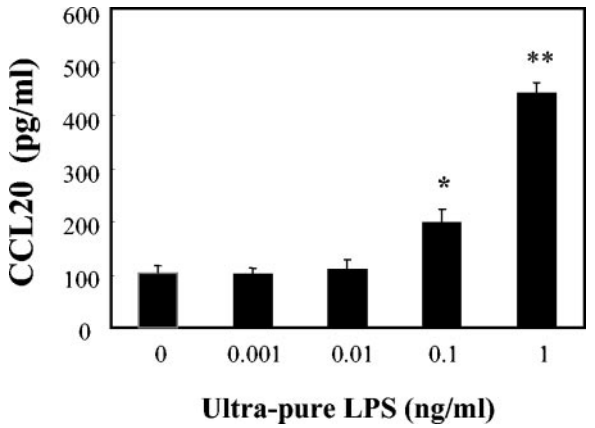

FIG. 3. Effect of repurified LPS on polarized uterine epithelial cell production of CCL20 and TNF-α. Uterine epithelial cells cultured in F12K complete medium on Nunc cell culture inserts were treated apically with ultrapure LPS in complete medium and then incubated for 24 h. After collection of the basolateral medium, cytokine levels were determined by ELISA. The values shown are means ± SE for groups of four to five wells. *, significantly different from control ($P < 0.05$); **, significantly different from control ($P < 0.01$). The data are representative of three separate experiments.

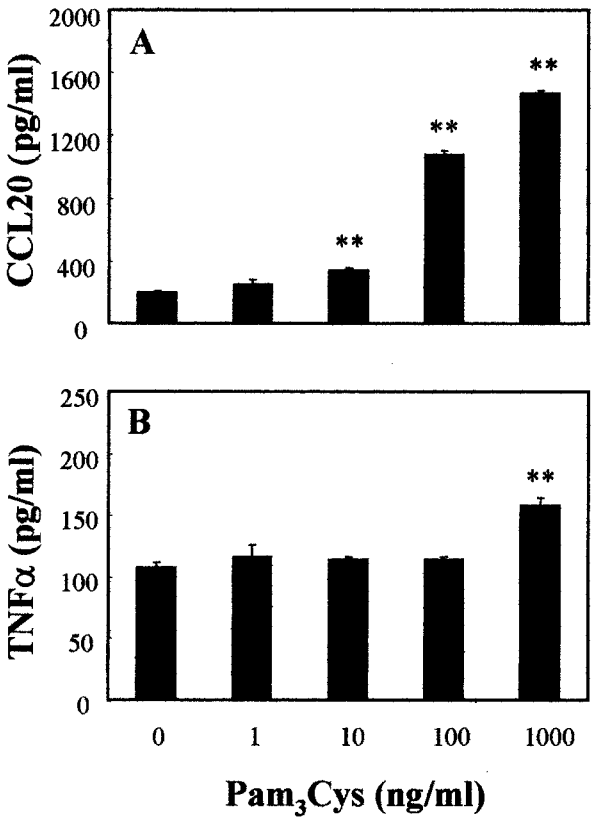

FIG. 4. Basolateral release of CCL20 (A) and TNF-α (B) by epithelial cells in response to increasing doses of Pam_3Cys. Primary rat uterine epithelial cells were grown to polarized monolayers on Nunc cell culture inserts and then treated apically with Pam_3Cys in complete F12K medium. The basolateral compartments received fresh complete F12K medium at the time of treatment. The basolateral medium was collected after a 24-h incubation. The values shown are means ± SE for four wells per group. **, significantly ($P < 0.01$) different from control cultures that were treated with medium without Pam_3Cys. The data are representative of three separate experiments.

To analyze the effects of a TLR2/1 agonist, we added Pam_3Cys, a known bacterial lipoprotein analog, to the apical surfaces of UEC prior to incubation for 24 h. As shown in Fig. 4, in the presence of increasing doses of Pam_3Cys, CCL20 release increased relative to that in control cells (Fig. 4A) without affecting the TER (data not shown). Interestingly, only the highest dose of Pam_3Cys used increased the amount of basolateral TNF-α (Fig. 4B).

Apical versus basolateral release of CCL20 and TNF-α by apically treated uterine epithelial cells. Previously, we showed that in unstimulated uterine epithelial cell cultures, CCL20 is preferentially released into the basolateral compartment while preferential TNF-α release is apical (8). To determine if directional release is altered in cell cultures treated with PAMP, we treated UEC apically with 1 μg of ultrapure LPS or Pam_3Cys/ml. As shown in Fig. 5A, both apical and basolateral CCL20 levels increased in response to PAMP treatment without altering the directional release seen with untreated UEC cultures.

Time course of CCL20 and TNF-α release by uterine epithelial cells stimulated with repurified LPS and Pam_3Cys. To determine the patterns of CCL20 and TNF-α release in response to repurified LPS and Pam_3Cys, we allowed rat uterine epithelial cells to grow to confluence on Nunc cell culture inserts for various times in the presence or absence of a PAMP. The cultures were treated apically with 1 μg of LPS or Pam_3Cys/ml. For this study, inserts containing epithelial cells were transferred to new wells containing fresh medium at 2-h intervals. As shown in Fig. 6A, LPS and Pam_3Cys had minimal effects on CCL20 during the first 2-h interval, after which the levels peaked between 2 and 4 h. While its level was partially elevated between 4 and 6 h, CCL20 then declined to baseline levels by 12 h. Figure 6B shows the cumulative pattern of release of CCL20, which indicates that cultures stimulated with Pam_3Cys release less CCL20 than cells stimulated with the same amount of LPS. In contrast, the release of TNF-α over time was gradual and was not significantly different from that seen in control wells, in part due to the limits of detection of our assay (data not shown).

CCL20 and TNF-α synthesis in response to treatment with actinomycin D and cell lysis studies. A study was undertaken to determine if CCL20 and TNF-α are synthesized by uterine epithelial cells in response to LPS. Primary cultures that had grown to confluence in Nunc cell culture inserts were treated in the apical compartment with 100 ng of ultrapure LPS/ml. Actinomycin D was added to the medium in both the apical and basolateral chambers at the time of addition of LPS. After 8 h of exposure, the basolateral medium was collected and analyzed for the presence of CCL20 and TNF-α. As shown in Fig. 7A, the release of CCL20 in response to LPS was completely inhibited at all doses of actinomycin D (1 to 100 μg/ml). In contrast, as indicated in Fig. 7B, TNF-α release in response to LPS was not affected by actinomycin D at 1 μg/ml but was gradually inhibited with increasing doses of actinomycin D (5 to 100 μg/ml).

To more fully define whether CCL20 and TNF-α were syn-

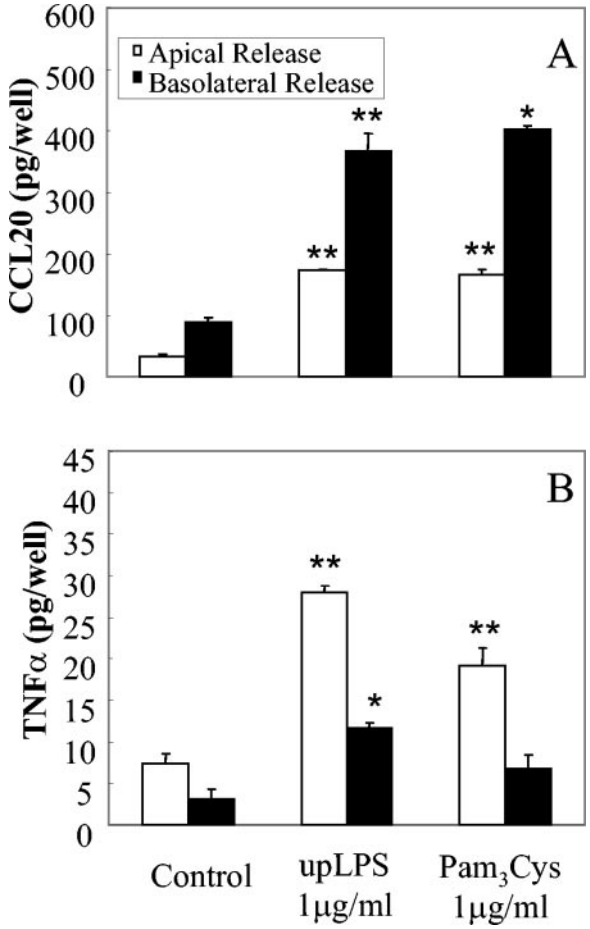

FIG. 5. Increased apical and basolateral release of CCL20 and TNF-α by uterine epithelial cells when stimulated apically with PAMPs. Uterine epithelial cells cultured in complete F12K medium on Nunc cell culture inserts were treated apically with 1 μg of ultrapure LPS or Pam_3Cys/ml and then incubated for 24 h. After collection of the apical and basolateral media, the cytokine levels were determined by ELISA. The values shown are means ± SE for groups of five wells. *, significantly different from the control for the same compartment ($P < 0.05$); **, $P < 0.001$. The data are representative of two separate experiments.

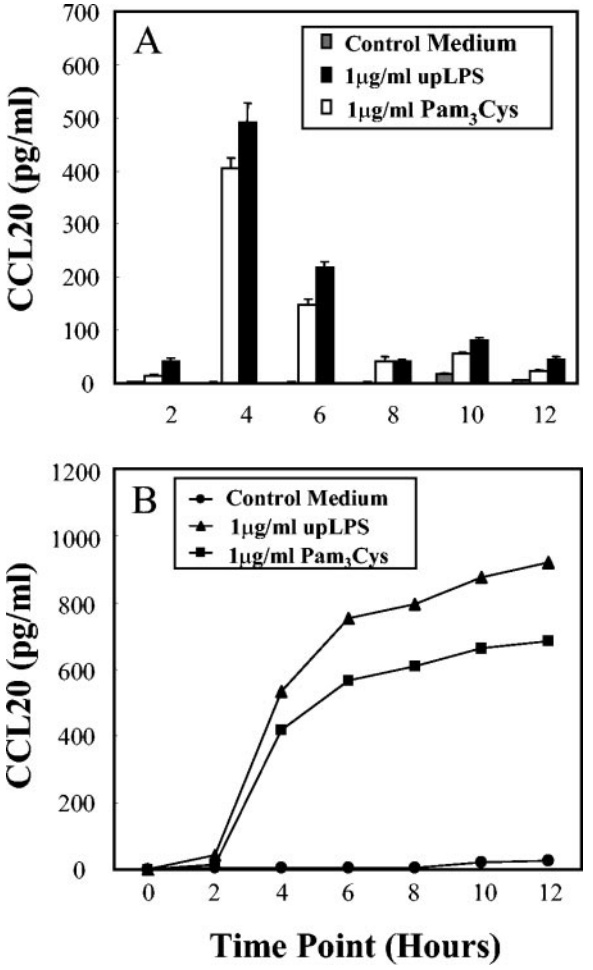

FIG. 6. (A) Time course of CCL20 release in response to ultrapure LPS and Pam_3Cys. (B) Cumulative pattern of CCL20 release over time. Polarized uterine epithelial cells grown on Nunc cell culture inserts in complete F12K medium were treated apically with 1 μg of ultrapure LPS or Pam_3Cys/ml. At the start of the experiment and at 2-h intervals thereafter, the culture inserts were moved to new wells containing fresh medium. The results, as determined by ELISA analysis of the basolateral medium, are representative of five wells per group in two separate experiments.

thesized by epithelial cells in response to LPS, we conducted cell lysis studies to evaluate directly whether epithelial cells contained sufficient intracellular CCL20 and TNF-α levels to account for their release into the culture medium. Polarized cell cultures were treated with fresh medium, with or without LPS. At time zero, some cultures were treated with fresh medium and removed for subsequent lysis by freezing or homogenization (PowerGen Tissue Homogenizer; Fisher Scientific). Other cultures were incubated for 8 h in the presence or absence of LPS, after which cells as well as the culture medium from apical and basolateral compartments were collected. The cells were then lysed by freezing or by homogenization as previously described (59). As shown in Fig. 8A and B, the epithelial cell contents of CCL20 and TNF-α at time zero were either close to or below the level of detection of our assay. When cells were incubated for 8 h, the amounts of CCL20 and TNF-α released into the medium were low but measurable. In contrast, when cells were incubated for 8 h with LPS prior to measuring the apical and basolateral media, significantly higher levels of CCL20 and TNF-α were found in the medium. Overall, these studies demonstrate that the amounts of CCL20 and TNF-α measured in cells prior to LPS treatment are insufficient to account for those released into the culture medium. These results, when considered along with our findings using actinomycin D, indicate that CCL20, and possibly TNF-α, is produced by UEC in response to LPS treatment.

DISCUSSION

The studies presented here demonstrate that polarized rat uterine epithelial cells are responsive to PAMPs. We showed that Pam_3Cys and LTA, which are specific ligands of TLR2, and LPS, which binds TLR4, stimulate the basolateral release of CCL20 and TNF-α by epithelial cells. Moreover, we showed

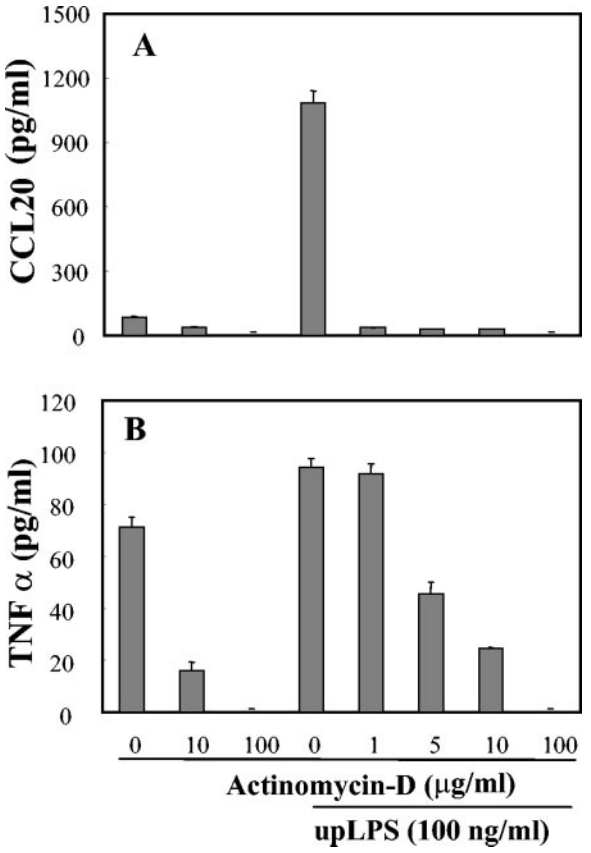

FIG. 7. Effect of actinomycin D on CCL20 (A) and TNF-α (B) synthesis. Rat uterine epithelial cells were grown on Nunc cell culture inserts in complete F12K medium prior to incubation in medium containing actinomycin D (apical and basolateral) and/or ultrapure LPS (apical). After an 8-h incubation, the basolateral medium was collected and analyzed by ELISA for CCL20 and TNF-α. The data are representative of four to five wells per group in two separate experiments.

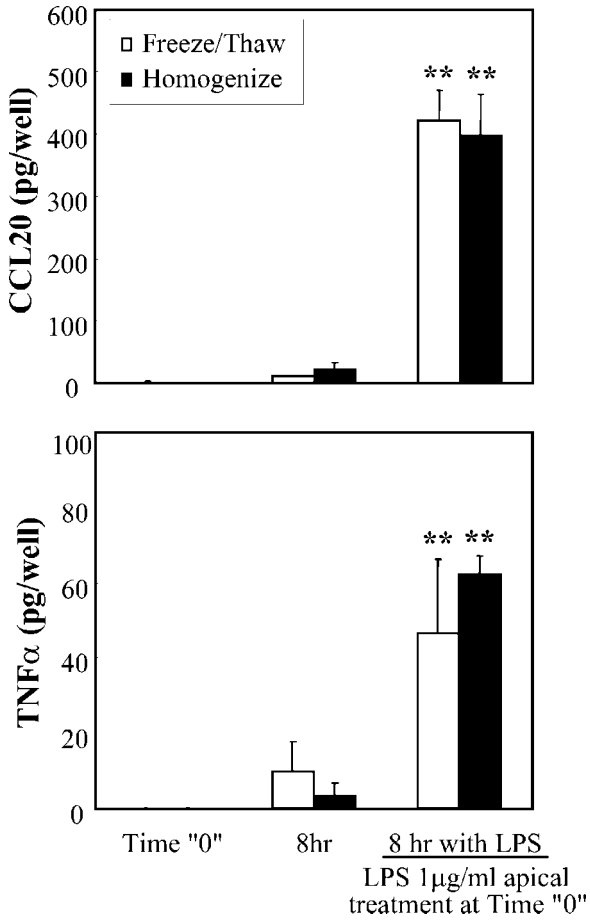

FIG. 8. Cell lysis studies of total CCL20 and TNF-α produced by LPS- or medium-treated cells. Polarized uterine epithelial cells were grown on cell culture inserts as previously described. At time zero, fresh medium was placed into the apical and basolateral compartments, and the cells were treated with LPS or medium alone. After an 8-h incubation, the apical and basolateral media were collected. Cell lysis at time zero and after 8 h was performed with fresh medium by a freeze-thaw method or by mechanical homogenization. The bars for the 8-h data show the total amounts of cytokine produced by each group, including apical and basolateral release. The zero time point analysis shows cell-associated CCL20 and TNF-α. The results are means ± SE for three to four wells per group and are representative of two separate experiments.

that the preferential release of TNF-α from the apical, or luminal, surfaces of UEC is maintained in response to PAMP stimulation, while CCL20 release, similar to a constitutive release pattern, is preferentially released to the basolateral, or stromal, surfaces of these cells. These studies demonstrate a rapid induction and basolateral release of CCL20 by primary polarized uterine epithelial cells between 2 and 4 h after apical treatment with LPS or Pam_3Cys. An actinomycin D treatment of cell cultures inhibited the release of CCL20 and TNF-α. When considered in conjunction with cell lysis studies showing the presence of only small amounts of intracellular CCL20 and TNF-α in the absence of LPS treatment, these studies suggest that CCL20 and TNF-α are synthesized by UEC in response to bacterial stimulation.

The basolateral cytokine release by uterine epithelial cells in response to LPS parallels the effects of treatment with *E. coli*. Previously, we demonstrated that both live and heat-killed *E. coli* placed at the apical or luminal surfaces of polarized primary rat uterine epithelial cells resulted in an increase in basolateral CCL20 and TNF-α release (8). The present study extends these findings by showing that treatment with LPS, a cell wall component of gram-negative bacteria, elicits a pattern of cytokine release similar to that seen when the cell cultures were stimulated with *E. coli*, a gram-negative species. In contrast to the case for *E. coli*, our previous studies showed that UEC exposure to *L. rhamnosus*, which is gram positive, has no effect on the release of either CCL20 or TNF-α. Unexpectedly, in the present study we found that LTA, a component of gram-positive bacterial cell walls, increased the levels of CCL20 and TNF-α. Moreover, Pam_3Cys, which like LTA is a ligand of TLR2, also increased CCL20 and TNF-α release. One explanation for the differences seen between *L. rhamnosus*, LTA, and Pam_3Cys may be that cell wall components of the live and heat-killed gram-positive bacteria used in previous experiments did not reach the surfaces of epithelial cells. This may be attributed to the strong cell wall of gram-positive bacteria such as lactobacilli, which are less prone to fragmentation

than cell walls from gram-negative bacteria such as *E. coli* (53). Alternatively, uterine epithelial cells are known to produce mucin and surfactants that act as physical or chemical barriers to prevent bacteria from interacting with epithelial cell receptors (2, 9, 36, 39). Purified antigens (PAMPs) such as LPS, LTA, and Pam_3Cys may pass through this barrier to interact with the cell surface receptors. Our studies suggest that once this barrier is breached, epithelial cells are able to recognize PAMPs of gram-negative as well as gram-positive bacteria to elicit an immune response. Studies are under way to determine whether differences seen with whole bacteria and PAMPs are due to bacterial exclusion.

Previous studies in our laboratory and by others have shown that the epithelium of the uterus, similar to epithelia at other mucosal sites, is supported by an underlying population of immune cells that are dispersed as well as present as lymphoid aggregates made up of a B-cell core surrounded by T cells with an outer halo of macrophages (27, 38, 60). The hypothesis that CCL20 production by epithelial cells plays a role in the recruitment of immune cells to the female reproductive tract was suggested by knockout studies of CCR6, the unique receptor for CCL20 (7, 55). In the absence of CCR6, Peyer's patches in the gut are underdeveloped, with dendritic cells being absent from the subepithelial dome. Our previous studies suggested that immune cell recruitment to the female reproductive tract may be due in part to the constitutive release of CCL20 (8). The present study further indicates that epithelial cells respond to PAMPs with a rapid release of CCL20, possibly to enhance further immune cell traffic to those sites in the uterus that are exposed to potential pathogens. Our findings of only small amounts of CCL20 and TNF-α in epithelial cells in the absence of stimulation (lysis and actinomycin D studies) suggest that in response to PAMPs, CCL20 and TNF-α are rapidly synthesized and released by UEC to mount a robust response under conditions that simulate infection. These findings, however, do not exclude the possibility that CCL20 and TNF-α exist as preformed cytokines whose processing under transcriptional control, since measurable TNF-α requires that membrane-anchored TNF-α precursors be released by proteolytic cleavage (15).

Others have demonstrated that commercial-grade LPS contains both LPS and lipoproteins (21). To more fully define the specificity of TLR signaling, Hirschfeld and colleagues developed a phenol extraction process to eliminate bacterial lipoproteins from LPS (21). The use of refined ultrapure LPS led to the conclusion that LPS binding signals through TLR4 and not through TLR2, which binds to bacterial lipoproteins and other ligands (3, 49). Our finding that repurified LPS stimulates the release of CCL20 supports our hypothesis that TLR4 is present and functional on uterine epithelial cells. Since epithelial cells respond to ultrapure LPS added to the apical surface, TLR4 may be present on the apical surfaces of rat uterine epithelial cells. An alternative interpretation of these findings is that PAMPs may move through epithelial cells to interact with TLRs at the basolateral surface. For example, in experiments with epithelial cell lines derived from gastrointestinal enterocytes, TLR4 was shown to be present only on the basolateral surface (1). Studies are under way to identify the sites on epithelial cells at which TLR2 and TLR4 are expressed.

We previously showed that polarized uterine epithelial cells in culture preferentially release TNF-α into the apical compartment and CCL20 into the basolateral compartment (8). Our findings in the present study extend these observations by showing that preferential release is not altered but rather is enhanced upon PAMP treatment. Also, irrespective of the PAMP tested, the TER, which is a measure of the UEC monolayer integrity (16), was not affected. The continuity of the TER, when coupled with the patterns of cytokine release in response to PAMPs as well as to live and heat-killed bacteria (8), suggests that in response to bacterial antigens, UEC are able to maintain an epithelial barrier and to signal to immune cells.

The movement of vaginal contents, including microbes and microbial debris, into the upper reproductive tract (32, 33) represents an ongoing challenge for the immune system in the reproductive tracts of mammals. Uterine innate and adaptive immune responses must balance the dual requirements of neutrality toward sperm and fetal or placental antigens and the need to recognize and respond to potentially pathogenic bacteria. Recent studies of the innate immune system indicate that beyond their barrier function, uterine epithelial cells release antimicrobial molecules, including β-defensins and SLPI (14, 30, 54). These molecules, along with the transport of IgA and the synthesis of complement (40, 41), provide a level of protection against bacteria that periodically enter the cervix, uterus, and fallopian tubes. CCL20 shares structural and functional characteristics with β-defensins (23, 48). CCL20 and β-defensins are antimicrobial, and both bind the CCR6 receptor. The recognition that UEC produce CCL20 demonstrates that the innate immune system in the uterus contains previously undefined antimicrobial agents that are potentially protective against gram-negative and gram-positive bacteria. Our findings of a dose-dependent release of CCL20 in response to Pam_3Cys and to ultrapure LPS suggest that the uterine epithelium has evolved to be responsive to pathogens at mucosal surfaces as well as to those that penetrate the epithelial lining. Our finding that CCL20 is produced (8) and stimulated in response to PAMPs suggests an important new arm of innate immune protection in the uterus. Whether PAMP-induced signaling stimulates β-defensin and SLPI production in addition to cell recruitment remains to be determined. What is clear is that the uterine epithelial cell response to PAMP is rapid, as determined by the pattern of CCL20 release. These findings suggest that in response to bacterial antigens, epithelial cells are able to mobilize immune protection (innate and adaptive) within hours of an antigenic challenge.

In conclusion, CCL20 and TNF-α are synthesized and secreted by uterine epithelial cells in response to stimulation by known agonists of TLRs. The responsiveness to these antigens suggests that the uterine epithelium plays a crucial role in alerting supportive mucosal immune cells to the presence of both commensal and pathogenic bacteria in the reproductive tract to fine tune the immune state in order to optimize conditions for successful mammalian reproduction.

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REFERENCES

1. **Abreu, M. T., L. S. Thomas, E. T. Arnold, K. Lukasek, K. S. Michelsen, and M. Arditi.** 2003. TLR signaling at the intestinal epithelial interface. J. Endotoxin Res. **9:**322–330.
2. **Akiyama, J., A. Hoffman, C. Brown, L. Allen, J. Edmondson, F. Poulain, and S. Hawgood.** 2002. Tissue distribution of surfactant proteins A and D in the mouse. J. Histochem. Cytochem. **50:**993–996.
3. **Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky.** 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science **285:**736–739.
4. **Becker, M. N., G. Diamond, M. W. Verghese, and S. H. Randell.** 2000. CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. J. Biol. Chem. **275:**29731–29736.
5. **Carramolino, L., L. Kremer, I. Goya, R. Varona, J. M. Buesa, J. Gutierrez, A. Zaballos, A. C. Martinez, and G. Marquez.** 1999. Down-regulation of the beta-chemokine receptor CCR6 in dendritic cells mediated by TNF-alpha and IL-4. J. Leukoc. Biol. **66:**837–844.
6. **Christ, A. D., and R. S. Blumberg.** 1997. The intestinal epithelial cell: immunological aspects. Springer Semin. Immunopathol. **18:**449–461.
7. **Cook, D. N., D. M. Prosser, R. Forster, J. Zhang, N. A. Kuklin, S. J. Abbondanzo, X. D. Niu, S. C. Chen, D. J. Manfra, M. T. Wiekowski, L. M. Sullivan, S. R. Smith, H. B. Greenberg, S. K. Narula, M. Lipp, and S. A. Lira.** 2000. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity **12:**495–503.
8. **Crane-Godreau, M. A., and C. R. Wira.** 2004. Effect of *Escherichia coli* and *Lactobacillus rhamnosus* on macrophage inflammatory protein 3 alpha, tumor necrosis factor alpha, and transforming growth factor beta release by polarized rat uterine epithelial cells in culture. Infect. Immun. **72:**1866–1873.
9. **DeSouza, M. M., S. K. Mani, J. Julian, and D. D. Carson.** 1998. Reduction of mucin-1 expression during the receptive phase in the rat uterus. Biol. Reprod. **58:**1503–1507.
10. **Drake, P. M., K. Red-Horse, and S. J. Fisher.** 2002. Chemokine expression and function at the human maternal-fetal interface. Rev. Endocr. Metab. Disord. **3:**159–165.
11. **Eckmann, L., M. F. Kagnoff, and J. Fierer.** 1995. Intestinal epithelial cells as watchdogs for the natural immune system. Trends Microbiol. **3:**118–120.
12. **Fahey, J. V., S. L. Humphrey, J. E. Stern, and C. R. Wira.** 1998. Secretory component production by polarized epithelial cells from the human female reproductive tract. Immunol. Investig. **27:**167–180.
13. **Fahey, J. V., R. H. Prabhala, P. M. Guyre, and C. R. Wira.** 1999. Antigen-presenting cells in the human female reproductive tract: analysis of antigen presentation in pre- and post-menopausal women. Am. J. Reprod. Immunol. **42:**49–57.
14. **Fahey, J. V., and C. R. Wira.** 2002. Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. J. Infect. Dis. **185:**1606–1613.
15. **Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. M. Clements, M. Crimmin, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, et al.** 1995. Matrix metalloproteinases and processing of pro-TNF-alpha. J. Leukoc. Biol. **57:**774–777.
16. **Glasser, S. R., J. Julian, G. L. Decker, J. P. Tang, and D. D. Carson.** 1988. Development of morphological and functional polarity in primary cultures of immature rat uterine epithelial cells. J. Cell Biol. **107:**2409–2423.
17. **Godefroy, S., G. Guironnet, C. Jacquet, D. Schmitt, and M. J. Staquet.** 2001. A combination of MIP-3alpha and TGF-beta1 is required for the attraction of human Langerhans precursor cells through a dermal-epidermal barrier. Eur. J. Cell Biol. **80:**335–340.
18. **Grant, K. S., and C. R. Wira.** 2003. Effect of mouse uterine stromal cells on epithelial cell transepithelial resistance (TER) and TNF-α and TGF-β release in culture. Biol. Reprod. **69:**1091–1098.
19. **Hajjar, A. M., D. S. O'Mahony, A. Ozinsky, D. M. Underhill, A. Aderem, S. J. Klebanoff, and C. B. Wilson.** 2001. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J. Immunol. **166:**15–19.
20. **Han, S. H., J. H. Kim, M. Martin, S. M. Michalek, and M. H. Nahm.** 2003. Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. Infect. Immun. **71:**5541–5548.
21. **Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis.** 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. J. Immunol. **165:**618–622.
22. **Hirschfeld, M., J. J. Weis, V. Toshchakov, C. A. Salkowski, M. J. Cody, D. C. Ward, N. Qureshi, S. M. Michalek, and S. N. Vogel.** 2001. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun. **69:**1477–1482.
23. **Hoover, D. M., C. Boulegue, D. Yang, J. J. Oppenheim, K. Tucker, W. Lu, and J. Lubkowski.** 2002. The structure of human macrophage inflammatory protein-3alpha/CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human beta-defensins. J. Biol. Chem. **277:**37647–37654.
24. **Janeway, C. A., Jr.** 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol. Today **13:**11–16.
25. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. Annu. Rev. Immunol. **20:**197–216.
26. **Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff.** 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Investig. **95:**55–65.
27. **Kaushic, C., E. Frauendorf, R. M. Rossoll, J. M. Richardson, and C. R. Wira.** 1998. Influence of the estrous cycle on the presence and distribution of immune cells in the rat reproductive tract. Am. J. Reprod. Immunol. **39:**209–216.
28. **Kayisli, U. A., N. G. Mahutte, and A. Arici.** 2002. Uterine chemokines in reproductive physiology and pathology. Am. J. Reprod. Immunol. **47:**213–221.
29. **Kim, J. M., L. Eckmann, T. C. Savidge, D. C. Lowe, T. Witthoft, and M. F. Kagnoff.** 1998. Apoptosis of human intestinal epithelial cells after bacterial invasion. J. Clin. Investig. **102:**1815–1823.
30. **King, A. E., D. C. Fleming, H. O. Critchley, and R. W. Kelly.** 2002. Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol. Hum. Reprod. **8:**341–349.
31. **Kunkel, E. J., D. J. Campbell, and E. C. Butcher.** 2003. Chemokines in lymphocyte trafficking and intestinal immunity. Microcirculation **10:**313–323.
32. **Kunz, G., D. Beil, H. Deiniger, A. Einspanier, G. Mall, and G. Leyendecker.** 1997. The uterine peristaltic pump. Normal and impeded sperm transport within the female genital tract. Adv. Exp. Med. Biol. **424:**267–277.
33. **Kunz, G., and G. Leyendecker.** 2002. Uterine peristaltic activity during the menstrual cycle: characterization, regulation, function and dysfunction. Reprod. Biomed. Online **4**(Suppl. 3)**:**5–9.
34. **Kwon, J. H., S. Keates, L. Bassani, L. F. Mayer, and A. C. Keates.** 2002. Colonic epithelial cells are a major site of macrophage inflammatory protein 3alpha (MIP-3alpha) production in normal colon and inflammatory bowel disease. Gut **51:**818–826.
35. **Liao, F., R. L. Rabin, C. S. Smith, G. Sharma, T. B. Nutman, and J. M. Farber.** 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. J. Immunol. **162:**186–194.
36. **Madsen, J., I. Tornoe, O. Nielsen, M. Lausen, I. Krebs, J. Mollenhauer, G. Kollender, A. Poustka, K. Skjodt, and U. Holmskov.** 2003. CRP-ductin, the mouse homologue of gp-340/deleted in malignant brain tumors 1 (DMBT1), binds gram-positive and gram-negative bacteria and interacts with lung surfactant protein D. Eur. J. Immunol. **33:**2327–2336.
37. **Medzhitov, R., and C. A. Janeway, Jr.** 1997. Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. **9:**4–9.
38. **Morris, H., J. Edwards, A. Tiltman, and M. Emms.** 1985. Endometrial lymphoid tissue: an immunohistological study. J. Clin. Pathol. **38:**644–652.
39. **Murakami, S., D. Iwaki, H. Mitsuzawa, H. Sano, H. Takahashi, D. R. Voelker, T. Akino, and Y. Kuroki.** 2002. Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-alpha secretion in U937 cells and alveolar macrophages by direct interaction with Toll-like receptor 2. J. Biol. Chem. **277:**6830–6837.
40. **Parr, E. L., and M. B. Parr.** 1988. Anti-bacterial IgA and IgG in mouse uterine luminal fluid, vaginal washings and serum. J. Reprod. Immunol. **13:**65–72.
41. **Parr, E. L., and M. B. Parr.** 1988. Deposition of C3 on bacteria in the mouse uterus after mating. J. Reprod. Immunol. **12:**315–319.
42. **Red-Horse, K., P. M. Drake, M. D. Gunn, and S. J. Fisher.** 2001. Chemokine ligand and receptor expression in the pregnant uterus: reciprocal patterns in complementary cell subsets suggest functional roles. Am. J. Pathol. **159:**2199–2213.
43. **Reibman, J., Y. Hsu, L. C. Chen, B. Bleck, and T. Gordon.** 2003. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. Am. J. Respir. Cell Mol. Biol. **28:**648–654.
44. **Reid, G., R. L. Cook, and A. W. Bruce.** 1987. Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. J. Urol. **138:**330–335.
45. **Richardson, J. M., C. Kaushic, and C. R. Wira.** 1995. Polymeric immunoglobin (Ig) receptor production and IgA transcytosis in polarized primary cultures of mature rat uterine epithelial cells. Biol. Reprod. **53:**488–498.
46. **Rotello, R. J., R. C. Lieberman, A. F. Purchio, and L. E. Gerschenson.** 1991. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor beta 1 in cultured uterine epithelial cells. Proc. Natl. Acad. Sci. USA **88:**3412–3415.
47. **Sato, K., H. Kawasaki, H. Nagayama, M. Enomoto, C. Morimoto, K. Tadokoro, T. Juji, and T. A. Takahashi.** 2000. TGF-beta 1 reciprocally controls chemotaxis of human peripheral blood monocyte-derived dendritic cells via chemokine receptors. J. Immunol. **164:**2285–2295.

48. **Schutyser, E., S. Struyf, and J. Van Damme.** 2003. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev. **14:**409–426.
49. **Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning.** 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. **274:**17406–17409.
50. **Sugarman, B. J., G. D. Lewis, T. E. Eessalu, B. B. Aggarwal, and H. M. Shepard.** 1987. Effects of growth factors on the antiproliferative activity of tumor necrosis factors. Cancer Res. **47:**780–786.
51. **Sugita, S., T. Kohno, K. Yamamoto, Y. Imaizumi, H. Nakajima, T. Ishimaru, and T. Matsuyama.** 2002. Induction of macrophage-inflammatory protein-3alpha gene expression by TNF-dependent NF-kappaB activation. J. Immunol. **168:**5621–5628.
52. **Sun, B., K. Nasu, J. Fukuda, S. Mine, M. Nishida, and I. Miyakawa.** 2002. Expression of macrophage inflammatory protein-3alpha in an endometrial epithelial cell line, HHUA, and cultured human endometrial stromal cells. Mol. Hum. Reprod. **8:**930–933.
53. **Tortora, G. J., B. R. Funke, and C. L. Case.** 1995. Microbiology, an introduction, 5th ed. The Benjamin/Cummins Publishing Company, Inc., Redwood City, Calif.
54. **Valore, E. V., C. H. Park, A. J. Quayle, K. R. Wiles, P. B. McCray, Jr., and T. Ganz.** 1998. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J. Clin. Investig. **101:**1633–1642.
55. **Varona, R., R. Villares, L. Carramolino, I. Goya, A. Zaballos, J. Gutierrez, M. Torres, A. C. Martinez, and G. Marquez.** 2001. CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. J. Clin. Investig. **107:**R37–R45.
56. **Wira, C. R., M. A. Roche, and R. M. Rossoll.** 2002. Antigen presentation by vaginal cells: role of TGFbeta as a mediator of estradiol inhibition of antigen presentation. Endocrinology **143:**2872–2879.
57. **Wira, C. R., and R. M. Rossoll.** 1995. Antigen-presenting cells in the female reproductive tract: influence of the estrous cycle on antigen presentation by uterine epithelial and stromal cells. Endocrinology **136:**4526–4534.
58. **Wira, C. R., and R. M. Rossoll.** 2003. Oestradiol regulation of antigen presentation by uterine stromal cells: role of transforming growth factor-beta production by epithelial cells in mediating antigen-presenting cell function. Immunology **109:**398–406.
59. **Wira, C. R., J. E. Stern, and E. Colby.** 1984. Estradiol regulation of secretory component in the uterus of the rat: evidence for involvement of RNA synthesis. J. Immunol. **133:**2624–2628.
60. **Yeaman, G. R., H. D. White, A. Howell, R. Prabhala, and C. R. Wira.** 1998. The mucosal immune system in the human female reproductive tract: potential insights into the heterosexual transmission of HIV. AIDS Res. Hum. Retrovir. **14**(Suppl. 1)**:**S57–S62.

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