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Polarized Epithelial Cells Secrete Interleukin 6 Apically in the Bovine Endometrium[1]

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ABSTRACT

Endometrial epithelial cells are the first line of defense against pathogenic bacteria infecting the uterus. Innate immune responses by these polarized epithelial cells to bacteria and tissue damage are characterized by release of the chemokine (C-X-C motif) ligand 8 (CXCL8) to attract immune cells from the circulation to the site of infection, where they are regulated by the cytokine interleukin (IL) 6. The present study tested the hypothesis that IL6 is predominantly secreted apically from polarized bovine endometrial epithelial cells in response to stimuli associated with bacterial infection and tissue damage. In postpartum animals, concentrations of IL6, but not of CXCL8, were higher in uterine mucus than in peripheral blood. In vitro, polarized endometrial epithelial cells only secreted IL6 apically when treated with bacteria, the pathogen-associated molecule lipopolysaccharide, or the damage-associated molecule IL1alpha, whereas CXCL8 accumulated apically and basolaterally. Furthermore, IL6 accumulated apically irrespective of whether lipopolysaccharide was applied to the apical or basolateral surface of epithelial cells. Secretion of IL6 from epithelial cells was dependent on the trans-Golgi network but was not affected by exogenous ovarian steroids or by coculture with stromal cells. However, a confluent epithelium was essential to protect underlying stromal cells against noxious challenges, including bacteria, lipopolysaccharide, IL1alpha, and a cytolysin. In summary, when a confluent endometrial epithelial cell barrier is faced with infection and damage, chemokines attract immune cells to the uterine lumen, but IL6 is solely secreted apically to ensure immune cells are only exposed to IL6 once they reach the lumen.

cell culture, cytokine, endometrium, epithelium, immunology, infection, inflammation, polarity, reproductive immunology, uterus

INTRODUCTION

Cell polarity is a structural feature of epithelial cells that can direct molecules toward their apical or basolateral surface [1, 2]. A polarized single columnar epithelium lines the uterine mucosa, with the apical surface of the cells facing the uterine lumen and the basal surface adjacent to the stroma. The apical and basolateral compartments of epithelia are separated by tight junctions between the cells [1, 2]. The endometrial epithelium is also the first line of defense in the uterus against microbes that ascend the female genital tract. Bacteria invade the uterus of cattle after parturition, during coitus, and even during pregnancy [3]. Postpartum infection of the endometrium leads to the recruitment of hematopoietic immune cells, particularly neutrophils, and the formation of pus, which is a characteristic sign of metritis or clinical endometritis [4]. These diseases delay the timing of insemination, reduce conception rates, and extend the interval from calving to conception [5].

A wide range of bacteria are isolated from the uterus of cattle or identified by genomic techniques, including gram-negative *Escherichia coli*, gram-positive *Trueperella pyogenes*, and anaerobic bacteria, such as *Fusobacterium*, *Bacteroides*, and *Prevotella* species [6–9]. Bacteria or their pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) from *E. coli*, are detected by innate immune receptors, such as Toll-like receptors (TLRs), on hematopoietic immune cells and endometrial cells. This sensing of pathogen-associated molecular patterns leads to cellular responses typified by release of chemokines, such as chemokine (C-X-C motif) ligand 8 (CXCL8; also known as interleukin [IL] 8) and chemokine (C-C motif) ligand 2 (CCL2), and cytokines, such as IL6, IL1β, tumor necrosis factor (TNF), and interferon gamma [10–12]. Bacteria also express virulence factors, such as the cholesterol-dependent cytolysin of *T. pyogenes*, pyolysin (PLO), which forms pores in the plasma membrane of cells, leading to cell death [13, 14]. Furthermore, cell damage combined with cellular responses to pathogens leads to the release of damage-associated molecular patterns, such as the intracellular cytokine IL1α, which stimulates further inflammation of endometrial cells via IL1 receptor type 1 (IL1R1) [15]. Endometrial epithelial cells collected from the postpartum bovine endometrium in vivo have increased *CXCL8* and *IL6* mRNA expression [16–18]. Similarly, ex vivo organ cultures and in vitro endometrial epithelial cell cultures release CXCL8 and IL6 protein in response to bacterial lipopeptides, LPS, and IL1α [12, 15, 19]. The CXCL8 protein was the founding member of the superfamily of chemokines, and it provides the chemotactic signal for immigration of neutrophils to sites of infection or tissue damage [20]. IL6 has a wide range of effects on hematopoietic immune cells, initially enhancing the recruitment of neutrophils to sites of infection [21, 22]. Later, IL6 acts to reduce the number of neutrophils and switch the pattern of immune cell infiltration toward monocytes [23]. We reasoned that when a confluent polarized epithelium encounters pathogens at the apical surface, CXCL8 would be secreted both apically and basolaterally to form a chemokine gradient to attract neutrophils from the vasculature, across the stroma, toward the epithelium, and into the uterine lumen. However, to avoid IL6 prematurely activating immune cells migrating along the CXCL8 gradient through the stroma, we hypothesized that IL6 might be directed preferentially toward the apical surface of the epithelium rather than basolaterally. Thus, the

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immigrating immune cells would only be activated by IL6 once they reached the site of infection in the uterine lumen.

Three-dimensional cell culture models allow polarized epithelial phenotypes to be replicated in vitro [2, 24–26]. Epithelial cells grown on cell culture inserts express tight junctions, and transepithelial electrical resistance (TER) increases when cells reach confluence. Once a confluent epithelium is formed, vectorial accumulation of molecules secreted from the epithelial cells can be explored by independently collecting the apical and basolateral cell culture supernatants. In support of our idea that IL6 may be directed apically during infection, polarized endometrial epithelial cells from mice and humans secrete more IL6 into the apical than the basolateral compartment in response to LPS [26, 27]. Although the apical and basolateral culture supernatants from polarized bovine endometrial epithelial cells treated with LPS are chemotactic for neutrophils [24], little is known about vectorial release of inflammatory mediators from the epithelium in cattle. Thus, the present study tested the hypothesis that IL6 is predominantly secreted apically from polarized endometrial epithelial cells in response to stimuli associated with bacterial infection and tissue damage.

MATERIALS AND METHODS

Collection of Samples from Animals

To examine female genital tract inflammatory responses, uterine mucus and peripheral blood were collected contemporaneously 3.5 ± 0.3 days (range, 2–6 days) postpartum from 44 Holstein-Friesian dairy cows across five farms. Experiments were performed in accordance with the guidelines and regulations, and with the ethical approval, of the U.K. Government Home Office (PPL 40/3478). Farm staff recorded whether dystocia or a normal puerperium was present. A 10-ml blood sample was collected from the coccygeal vein into an evacuated sterile tube (Vacutainer; BD) and allowed to clot at 4°C for 6 h before centrifugation at 1000 × *g* for 10 min to harvest serum, which was stored at −20°C. Mucus was collected using a gloved hand inserted through the vulva and into the vagina and was processed as described previously, placing 2 g of mucus in 10 ml of CytoLyt solution (40% methanol:60% distilled water; Cytyc Corporation) and mixed with 0.1% dithiothreitol (Sigma-Aldrich) until the mucus was disrupted, followed by centrifugation at 3000 × *g* for 15 min and collection of the supernatant [28]. The serum and mucus concentrations of IL6 and CXCL8 were measured by ELISA (see below).

Isolation and Culture of Endometrial Cells

Endometrial epithelial and stromal cell populations were isolated and cultured as described previously [12, 19, 24, 29]. Uteri were collected from female mixed-breed beef cattle (age, 2.2 ± 0.3 yr) within 15 min of slaughter during the normal work of a commercial slaughterhouse. The stage of reproductive cycle was determined by examination of ovarian morphology and vasculature [19, 29], and uteri at Days 1–4 of the estrous cycle were selected because plasma progesterone concentrations are basal [30]. The endometrium was enzymatically digested, and epithelial and stromal cells were separated by differential adhesion to cell culture flasks. Cells were cultured in culture medium comprising RPMI-1640 medium, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B (all from Sigma-Aldrich), with 10% heat-inactivated FBS (Biosera), incubated at 37°C in a humidified atmosphere of air with 5% CO_2, with the medium changed every 48 h. Epithelial and stromal cell populations were distinguished by cell morphology, and absence of immune cell contamination was confirmed by fluorescence-activated cell sorting analysis of CD45, vimentin, and cytokeratin [12].

Polarized epithelial cells were prepared by seeding 3×10^5 cells on each Millicell hanging cell culture insert (Millipore) already coated with 50 μl of Matrigel (BD Biosciences) diluted 1:8 in RPMI 1640, which does not impede movement of inflammatory mediators. Inserts were placed in 24-well plates (TPP; Helena Biosciences), with 300 and 800 μl of culture medium in the apical and basolateral compartment, respectively, as described previously [24]. Confluence of the epithelial layer was determined by TER > 1000 Ωcm^2, measured daily with an epithelial volt-ohm meter ($EVOM^2$; World Precision Instruments) using electrodes placed in the apical and basolateral compartments [24]. Unless specified otherwise, the polarized epithelial cells on cell culture inserts were treated after 7 ± 1 days of culture, once TER > 1000 Ωcm^2. For some studies, 1×10^5 stromal cells were seeded into the basolateral compartment of 24-well culture plates, below the epithelial cells in cell culture inserts or cell-free inserts [24].

All experiments were repeated on at least three independent occasions using two replicate wells for each treatment and with treatments applied for 24 h based on the results of previous studies [19, 31]. At the end of each experiment, TER was measured, the apical and basolateral supernatants collected and stored at −20°C for measurement of inflammatory mediators by ELISA, and cell viability assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [24, 32]. Briefly, cells were incubated for 1 h in culture medium containing 1 mg/ml of MTT, before lysis with dimethyl sulfoxide, and optical density (OD_{570}) was measured using a microplate reader (POLARstar Omega; BMG Labtech).

Cell Treatments

The main challenges facing endometrial cells during the first week postpartum are *E. coli* and the bacterium's LPS [33], the cholesterol-dependent cytolysin (PLO) from *T. pyogenes* [13], and the intracellular cytokine IL1α that acts as a damage-associated molecular pattern once released from damaged cells [15]. Endometrial pathogenic *E. coli* isolate MS499 [33, 34] was grown overnight in Luria-Bertani medium (Sigma-Aldrich), centrifuged at 6000 × *g* for 10 min at 4°C, washed in sterile PBS (Life Technologies Ltd.), and resuspended in culture medium. To generate heat-killed *E. coli*, 1×10^7 CFU/ml were incubated at 70°C for 30 min, washed in PBS, and then suspended at the equivalent of 1×10^7 CFU/ml in culture medium as described previously [13]. Ultrapure LPS from *E. coli* 0111:B4 was obtained from Invivogen and recombinant bovine IL1α from 2BScientfic. Recombinant PLO was a gift from Prof. H. Jost (Office for Research & Discovery, University of Arizona, Tucson, AZ) [35]. Ovarian and glucocorticoid steroids (estradiol, E2758; progesterone, P8783; dexamethasone, D4902) and were obtained from Sigma-Aldrich.

Cellular Inflammatory Responses

To evaluate cellular responses to bacteria that commonly ascend into the uterine lumen, polarized epithelial cells or cocultures of polarized epithelial cells above stromal cells were treated in the apical compartment with control medium or with medium containing 1×10^3 CFU/ml of live *E. coli*, an equivalent of 1×10^7 CFU/ml of heat-killed *E. coli*, or 100 ng/ml of LPS.

To examine epithelial cell responses to likely challenges that these cells experience in the uterine lumen during the puerperium in more molecular detail, polarized epithelial cells were treated in the apical compartment with control medium or with medium containing a 10-fold increasing concentrations of the pathogen-associated molecule LPS (1–1000 ng/ml), the bacterial cyolysin PLO (0.3–300 HU/ml), or the damage-associated molecule IL1α (0.1–100 ng/ml) based on previously validated concentrations [13, 15, 19].

Whereas most microbes ascend into the uterus via the cervix to impinge on the apical surface of the epithelium, systemic infections mean that microbes may also reach the basolateral surface of the endometrial epithelium. To examine whether vectorial accumulation of inflammatory mediators was directed toward the site of microbial challenge, polarized epithelial cells were treated with 100 ng/ml of LPS in the apical compartment, the basolateral compartment, or both.

Impact of Steroids on Inflammatory Responses

Ovarian steroids regulate the physiology of the endometrium, and progesterone increases the severity of endometritis in vivo [36]. To examine if ovarian steroids modulated epithelial cell function, polarized epithelial cells were incubated for 24 h in control medium or with medium containing 5 ng/ml of progesterone, 3 pg/ml of estradiol, or a combination of progesterone and estradiol. The steroid concentrations of progesterone and estradiol reflect serum concentrations during the luteal and follicular phases of the estrous cycle, respectively [9, 37]. After 24 h of treatment with steroids, the cells were challenged in the apical compartment only with control medium or with medium containing 100 ng/ml of LPS or 10 ng/ml of IL1α for a further 24 h; PLO was not used as PLO did not stimulate inflammation.

Dexamethasone is the prototypical glucocorticoid anti-inflammatory steroid and is effective with endometrial cells [38, 39]. Thus, to examine if dexamethasone modulated inflammatory responses of polarized epithelial cells, cells were incubated for 24 h in control medium or in medium containing 5 ng/ml of dexamethasone. After 24 h, cells were challenged in the apical

compartment with control medium or with medium containing 100 ng/ml of LPS or 10 ng/ml of IL1α for a further 24 h.

Trans-Golgi Network

Immune cells accumulate IL6 in the Golgi complex, which is released to the surface of cells when stimulated with LPS [40]. To examine the mechanisms of vectorial secretion of IL6 in endometrial epithelial cells and whether this utilized the trans-Golgi network, Brefeldin A (Sigma) was used to disrupt the structure and secretory function of the Golgi complex [40]. Polarized epithelial cells were incubated for 6 h in control medium or with medium containing 100 ng/ml of Brefeldin A according to the manufacturer's instructions. Then, the cells were challenged in the apical compartment with control vehicle or with 100 ng/ml of LPS for 6 h as, in preliminary experiments, 12-h treatment with Brefeldin A reduced cell viability.

Epithelial Integrity

The separation of the apical and basolateral compartments of mucosa depends on an intact sheet of epithelial cells. To explore whether integrity of the polarized epithelial layer was required for vectorial IL6 secretion, epithelial cells in cell culture inserts were examined before and after confluence. Epithelial cell TER was measured every 24 h from 2 to 9 days of culture. Cells were treated before and after 1000 Ωcm^2 TER on Days 2 and 9 of culture, respectively.

To further determine the importance of epithelial integrity, we exploited knowledge that stromal cells are more sensitive than epithelial cells to cytolysis caused by PLO [13]. Thus, confluent polarized epithelial cells or cell-free culture inserts were placed above stromal cells in the compartment below, and then the apical compartment was treated with control medium or with medium containing 0.3, 3, 30, or 300 HU/ml of PLO. After 24 h, stromal cell viability was measured using the MTT assay to evaluate cell viability, which is a means of evaluating PLO-mediated cytolysis [13, 14].

Finally, the functional value of epithelial integrity for protecting stromal cells against pathogen- or damage-associated molecules was examined. Polarized epithelial cells were cocultured with stromal cells in the compartment below, and the apical compartment was treated with control medium or with medium containing 100 ng/ml of LPS or 10 ng/ml of IL1α.

Enzyme-Linked Immunosorbent Assay

Concentrations of IL1β, IL6, TNF, interferon gamma, and CCL2 in cell culture supernatants were measured by ELISA according to the manufacturer's instructions (Bovine IL-1 beta ELISA Reagent Kit ESS0027 and Bovine IL-6 ELISA Reagent Kit ESS0029 from Thermo Scientific, Bovine TNF-alpha DuoSet DY2279 and Bovine IFN-gamma DuoSet DY2300 from R&D Systems Europe Ltd., and Bovine CCL2 ELISA, DIY0659B-003, from Kingfisher Biotech, Inc.). The concentrations of CXCL8, CXCL1, CXCL2, and CXCL3 were measured by ELISA as described previously [41, 42]. The inter- and intra-assay coefficients of variation were all less than 10%; the limits of detection were 13 pg/ml for IL1β, 75 pg/ml for IL6, 83 pg/ml for TNF, 200 pg/ml for interferon gamma, 75 pg/ml for CCL2, 65 pg/ml for CXCL8, 200 pg/ml for CXCL1, 100 pg/ml for CXCL2, and 150 pg/ml for CXCL3.

Statistical Analysis

Data are presented as the arithmetic mean ± SEM. For polarized epithelial cell experiments, the ELISA data are reported as picograms of inflammatory mediators that accumulated in each compartment (apical and basolateral). Statistical analyses were performed using SPSS 16.0 (SPSS, Inc.), with the animal as the designated statistical unit and $P < 0.05$ considered statistically significant. Treatments were compared using ANOVA with the Bonferroni or Dunnett post hoc test.

RESULTS

Directed Secretion of Inflammatory Mediators

The uterine lumen of dairy cattle is always contaminated with bacteria during the first week postpartum, and dystocia increases the risk of disease [9, 43]. To seek in vivo evidence for directed secretion of inflammatory mediators into the uterine lumen, the concentrations of IL6 and CXCL8 were measured in uterine mucus and peripheral serum collected contemporaneously from animals during the first week postpartum. The concentrations of IL6 were about 3-fold higher in mucus than in serum (Fig. 1A), whereas the concentrations of CXCL8 were similar in mucus and serum irrespective of normal parturition or dystocia (Fig. 1B). These observations support the notion that the endometrium may respond to infection or damage by vectorial release of spcific inflammatory mediators.

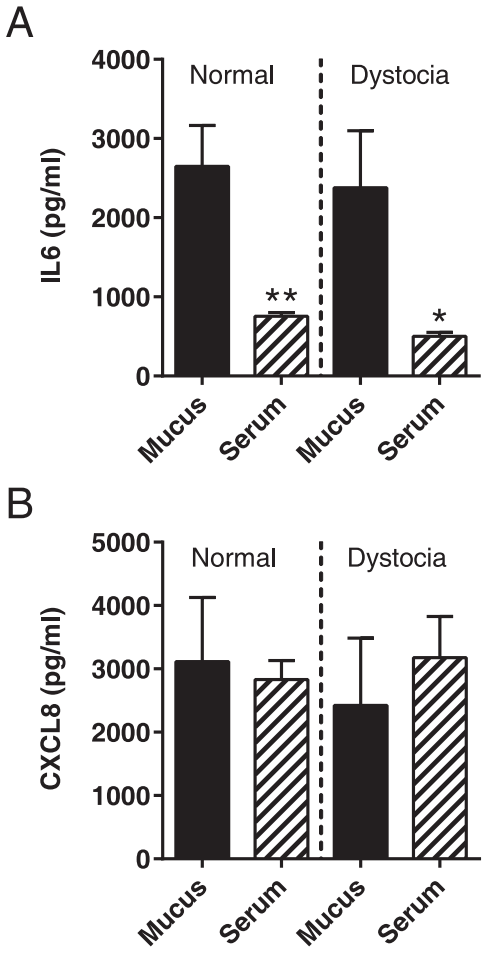


FIG. 1. Postpartum inflammatory responses in vivo. Uterine mucus and peripheral blood serum were collected during the first week postpartum from dairy cows that had a normal parturition (n = 33) or dystocia (n = 11), and IL6 (**A**) and CXCL8 (**B**) were measured by ELISA. Data are presented as the mean + SEM and were analyzed by ANOVA using the Bonferroni multiple comparison *t*-test. $*P < 0.05$, $**P < 0.01$ vs. mucus.

Vectorial Secretion of IL6 in Response to Bacteria

To explore whether vectorial secretion of inflammatory mediators occurs in response to microbes in vitro, polarized epithelial cells were treated apically with live endometrial pathogenic *E. coli*, heat-killed *E. coli*, or *E. coli* LPS. The supernatants in the apical compartment accumulated IL6, but no IL6 was detected in the basolateral compartment (Fig. 2A). However, CXCL8 accumulated in both the apical and basolateral compartments in response to each of the treatments (Fig. 2B). The TER was typical of a confluent epithelium, except for cells challenged with live *E. coli* (Fig. 2C), and no significant effect of treatment on epithelial cell viability was found (Fig. 2D). Microbiological cultures of the supernatant from the apical and the basolateral compartments yielded *E. coli* colonies for cells challenged with live but not with heat-killed *E.* coli, confirming the invasive nature of live endometrial

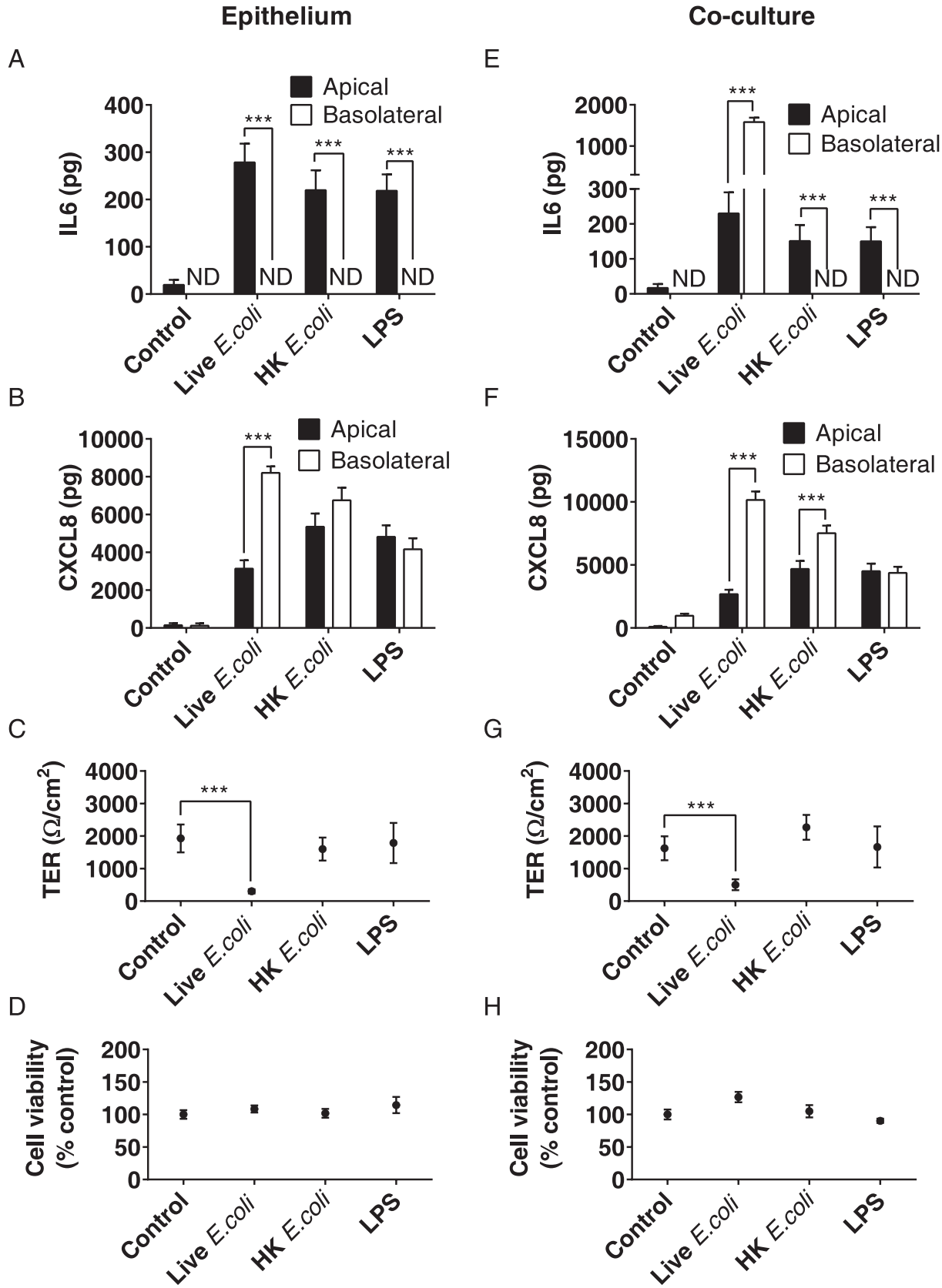


FIG. 2. Inflammatory responses to bacteria in vitro. Polarized endometrial epithelial cells on cell culture inserts, cultured alone (**left**) or cocultured above stromal cells (**right**), were treated for 24 h in the apical compartment with control medium, with medium containing live or heat-killed (HK) *E. coli*, or LPS. The concentrations of IL6 (**A** and **E**) and CXCL8 (**B** and **F**) were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars) compartments after TER was recorded (**C** and **G**) and cell viability evaluated by MTT assay (**D** and **H**). Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA using the Bonferroni multiple comparison *t*-test. ***$P < 0.001$. ND = below limits of detection.

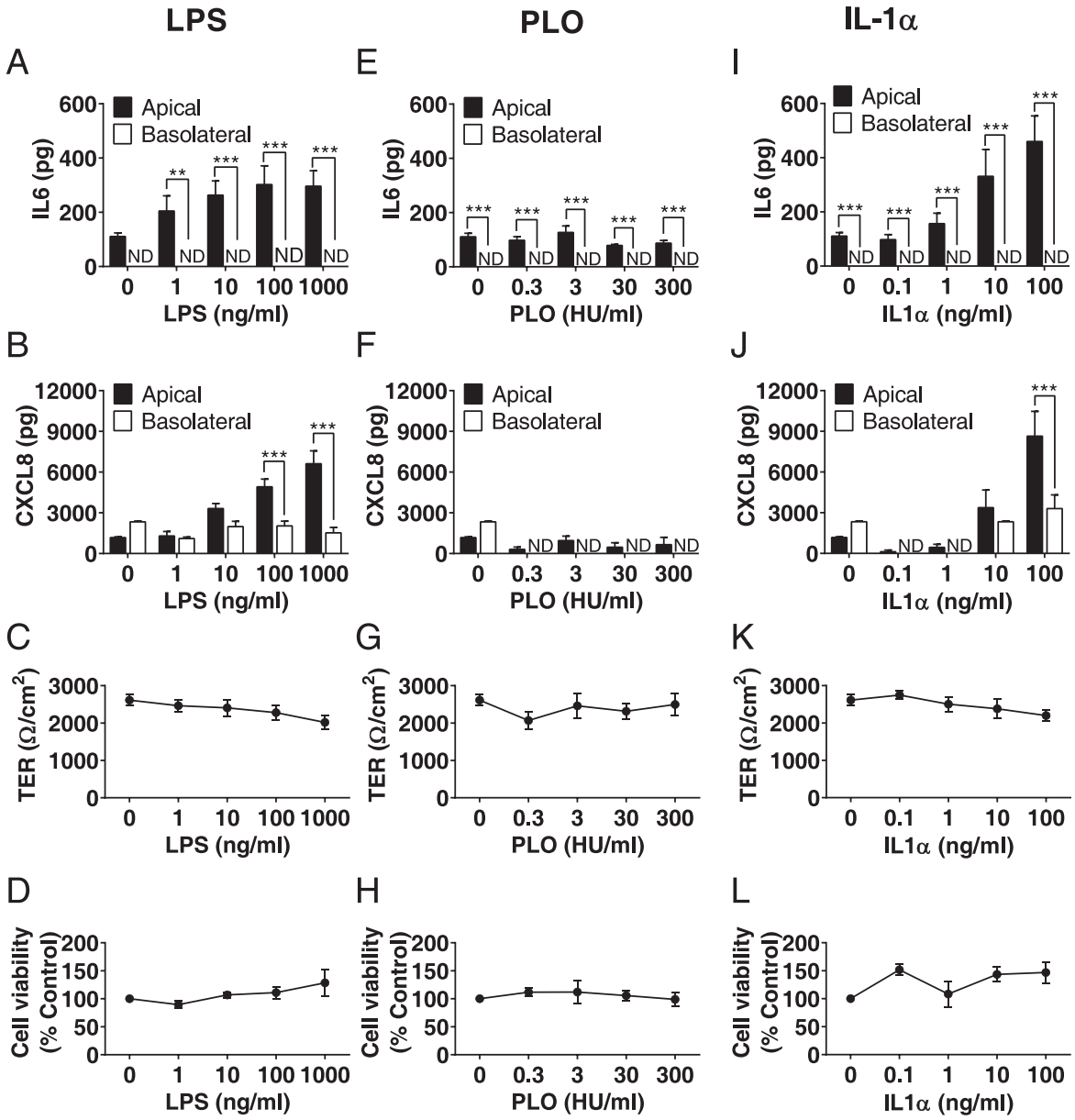


FIG. 3. Polarized epithelial cell inflammatory responses. Polarized epithelial cells on cell culture inserts were treated in the apical compartment for 24 h with control medium or with medium containing the indicated concentrations of the pathogen-associated molecular pattern (LPS; **left**), the cholesterol-dependent cytolysin (PLO; **middle**), or the damage-associated molecular pattern (IL1α; **right**). The concentrations of IL6 (**A**, **E**, and **I**) and CXCL8 (**B**, **F**, and **J**) were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars) compartments after TER was recorded (**C**, **G**, and **K**) and cell viability evaluated by MTT assay (**D**, **H**, and **L**). Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA with Bonferroni post hoc tests. $^{**}P < 0.01$, $^{***}P < 0.001$. ND = below limits of detection.

pathogenic *E. coli* [33]. Taken together, these results provide evidence that confluent polarized epithelial cells vectorially secrete IL6 in response to bacteria.

To explore whether coculture of epithelial cells with stromal cells may impact cellular responses, polarized epithelial cells on cell culture inserts were placed above stromal cells cultured on the surface of 24-well culture plates. Cell supernatants only accumulated IL6 in the apical compartment when epithelial cells were treated apically with LPS or heat-killed *E. coli*, but IL6 accumulated in both compartments when treated apically with live *E. coli* (Fig. 2E) and, as previously, CXCL8 accumulated in both compartments in response to all treatments (Fig. 2F). As before, bacteria were only cultured from the apical and basolateral compartments when cells were treated with live *E. coli*. Furthermore, the TER was only reduced when cells were treated with live *E. coli* (Fig. 2G), and no significant effect on epithelial cell viability was found (Fig. 2H). These observations provide evidence that stroma have little influence on the vectorial nature of IL6 secretion from polarized epithelial cells.

Vectorial Secretion of IL6 in Response to Pathogen and Damage Molecules

Endometrial epithelial cells are exposed to pathogen-associated molecular patterns, such as LPS; to bacterial virulence factors, such as the cholesterol-dependent cytolysin PLO; and to damage-associated molecules, such as IL1α [13, 15, 44]. Polarized epithelial cell supernatants treated apically with LPS accumulated IL6 in the apical compartment in a

concentration-dependent manner ($P < 0.001$), but no IL6 was detected in the basolateral compartment (Fig. 3A). However, CXCL8 was detected in both compartments, although a significant response to LPS was evident only in the apical compartment ($P < 0.001$) (Fig. 3B). No significant effect of LPS treatment on epithelial integrity as determined by TER measurement (Fig. 3C) or on cell viability as evaluated by MTT assay (Fig. 3D) was found. When polarized epithelial cells were treated apically with PLO, no significant increase in the accumulation of IL6 or CXCL8 was observed. Again, however, IL6 was not detected in the supernatants from the basolateral compartment (Fig. 3, E and F), and no effect on TER or cell viability (Fig. 3, G and H) was found. Apical treatment of epithelial cells with IL1α stimulated a concentration-dependent accumulation of IL6 and CXCL8 in the apical compartment ($P < 0.001$) (Fig. 3, I and J). As before, CXCL8, but not IL6, was detected in the basolateral compartment, and no significant effect on TER or cell viability was found (Fig. 3, K and L). The implications from these data are that inflammatory responses were associated with LPS and IL1α, but not with PLO, and that IL6 was secreted apically from the epithelium.

IL6 Is Uniquely Secreted Apically

To determine if the vectorial accumulation of IL6 was a generalized mechanism, polarized epithelial cells were treated with LPS and cell supernatants collected to measure the concentrations of a range of inflammatory mediators. In addition, cells were treated with LPS in the apical compartment, the basolateral compartment, or both to determine if the location of challenge impacted the direction of secretion of inflammatory mediators. Treatment with LPS apically or in both compartments simulated the accumulation of IL6 ($P < 0.001$) but only in the apical compartment (Fig. 4A). Increased concentrations of inflammatory mediators in response to LPS were also evident for CXCL8 (Fig. 4B), CXCL1 (Fig. 4C), CXCL2 (Fig. 4D), and CXCL3 (Fig. 4E), but not for CCL2 (Fig. 4F). However, CXCL8, CXCL1, CXCL2, CXCL3, and CCL2 were detectable in the basolateral compartment as well as in the apical compartment. The concentrations of TNF, IL1β, and interferon gamma were all below the detection limits of the assays. We conclude from these data that the vectorial inflammatory response to LPS by polarized epithelial cells appears to be specific for IL6 and not a generalized feature of other inflammatory mediators.

Effect of Steroids on Vectorial Secretion of IL6

Because endometrial cell functions may be modulated by ovarian steroid hormones [36], polarized epithelial cells were treated for 24 h with progesterone, estradiol, or a combination of progesterone and estradiol before challenge with LPS or IL1α in the apical compartment. The apical supernatants of the cells accumulated IL6 in response to LPS, but IL6 was not detected basolaterally as previously (Fig. 5A). However, treatment with steroids did not significantly affect the accumulation of IL6 (Fig. 5A) or the integrity of the epithelium as determined by TER concentrations (Fig. 5B). Dexamethasone is a steroid with established anti-inflammatory effects, so to confirm that polarized epithelial cells were responsive to steroid hormones, cells were treated with dexamethasone before challenge with LPS in the apical compartment. Dexamethasone reduced the accumulation of IL6 in the apical compartment in response to either challenge (Fig. 5C), although no accumulation of IL6 was detectable basolaterally and dexamethasone did not significantly affect TER (±9% of control). Similarly, ovarian steroids did not modulate the IL6 responses of epithelial cells to IL1α (Fig. 5, D and E), although dexamethasone did reduce the accumulation of IL6 (Fig. 5F). These data indicate that whereas epithelial cells respond to dexamethasone, little evidence was found for an effect of the ovarian steroids under the conditions of the present in vitro study.

Vectorial Secretion of IL6 via the Trans-Golgi Network

Many proteins, including cyotkines, are sorted and then secreted via the trans-Golgi network in epithelial cells [2]. To explore the mechanistic role of the trans-Golgi network for IL6 secretion, polarized epithelial cells were treated with Brefledin A, which blocks trans-Golgi transport [40]. Treatment with Brefeldin A reduced the accumulation of IL6 in response to challenge with LPS for 6 h (Fig. 6A) but did not lead to detection of IL6 basolaterally. Brefeldin A reduced the TER ($P < 0.05$) (Fig. 6B) but did not significantly affect cell survival (Fig. 6C). These data support the concept that IL6 is secreted via the trans-Golgi network.

Impact of Epithelial Integrity

To examine the importance of the integrity of the epithelium, epithelial cells were treated apically with LPS after 2 and 9 days on cell culture inserts, reflecting an incomplete and a confluent epithelium, respectively, which was confirmed by measuring TER (Fig. 7A). Treatment of epithelial cells with LPS before confluence increased the accumulation of IL6 apically ($P < 0.05$), and IL6 was detectable in both the apical and basolateral compartments (Fig. 7B). However, whereas LPS also stimulated the accumulation of IL6 apically with a confluent epithelium ($P < 0.001$), no LPS was detectable in the basolateral compartment.

To examine the functional importance of a confluent epithelium, PLO was added to the apical compartment of cocultured polarized epithelial cells above stromal cells or empty well inserts. The presence of polarized epithelial cells protected the stromal cells from lysis caused by PLO, whereas in the absence of epithelial cells, stromal cell lysis was evident with 30 HU of PLO or greater (Fig. 7C).

To further test the functional role of the epithelium, LPS or IL1α was added to the apical compartment of cocultured polarized epithelial cells above stromal cells. Because modest accumulation of IL6 was noted over 24 h even for cells in control medium, data are presented as the additional accumulation of IL6 above the endogenous IL6 secretion measured in the control treatment; this allowed determination of whether accumulation of IL6 in the basolateral compartment of cocultured stromal cells responded to apical challenges of polarized epithelial cells with LPS or IL1α. As previously observed, IL6 accumulated in the apical compartment in response to LPS or IL1α and in a concentration-dependent manner ($P < 0.001$). However, IL6 did not accumulate in the basolateral compartment when an intact confluent epithelium was present (Fig. 7, D and E). Furthermore, empty cell culture inserts were used as further controls to evaluate stromal cell responses when exposed to LPS or IL1α in the absence of protective epithelium. Indeed, in the absence of epithelial cells, an abundant and concentration-dependent accumulation of IL6 from stromal cells occurred when the apical compartment of cell culture inserts were treated with LPS or IL1α (hatched bars in Fig. 7, D and E). Taken together, these data provide evidence for the protective role of a confluent epithelium.

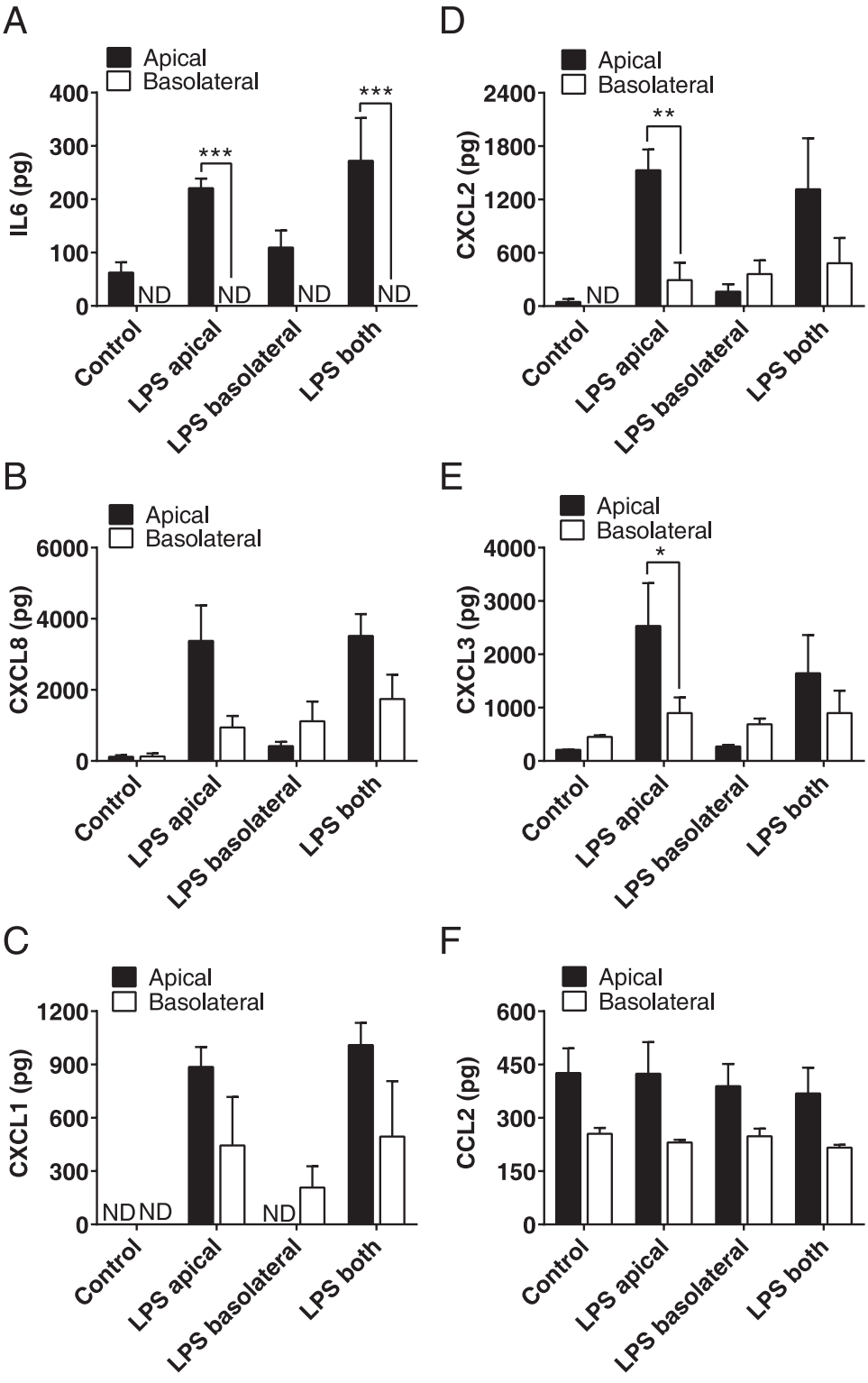


FIG. 4. Polarized epithelial cell inflammatory responses to LPS. Polarized epithelial cells on cell culture inserts were treated in the apical compartment, the basolateral compartment, or both for 24 h with control medium or with medium containing 100 ng/ml of LPS. Cell culture supernatants were collected independently from the apical (solid bars) and basolateral (open bars) compartments, and the concentrations of IL6 (**A**), CXCL8 (**B**), CXCL1 (**C**), CXCL2 (**D**), CXCL3 (**E**), and CCL2 (**F**) were measured by ELISA. Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA with Bonferroni post hoc tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ND = below limits of detection.

DISCUSSION

Ascending bacterial infections of the female genital tract, along with tissue damage, commonly cause uterine disease in cattle after parturition [7, 9, 45]. In the present study, the postpartum accumulation of IL6 in vivo appeared to be more toward the uterine lumen than peripheral blood. This led us to examine the vectorial secretion of inflammatory mediators from polarized endometrial epithelial cells. The most striking observation was that only IL6 consistently accumulated

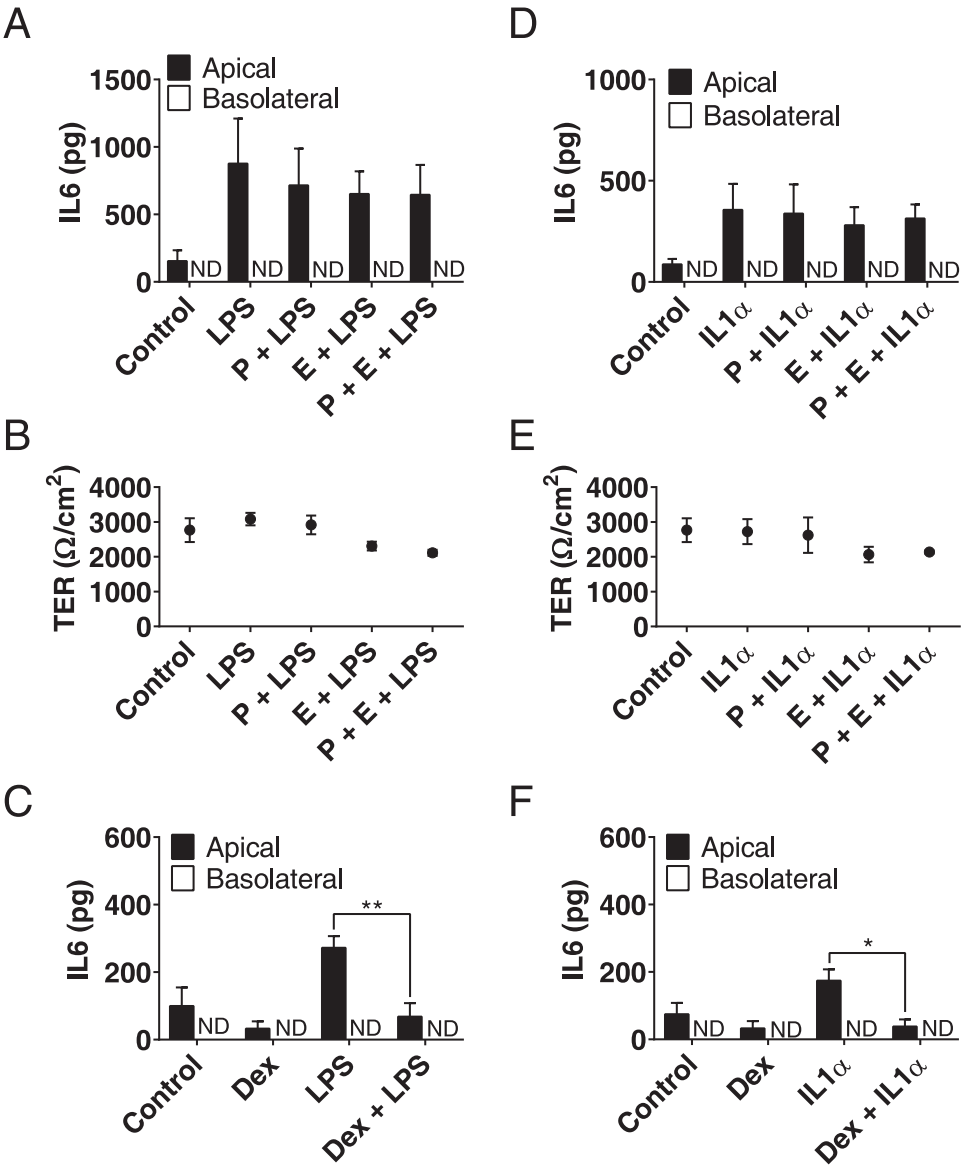


FIG. 5. Hormones and polarized epithelial cell inflammatory responses. Polarized epithelial cells on cell culture inserts were treated for 24 h with control medium or with medium containing 5 ng/ml of progesterone, 3 pg/ml of estradiol, or a combination of progesterone and estradiol (**A**, **B**, **D**, and **E**) or with 5 ng/ml of dexamethasone (**C** and **F**), followed by 24 h of treatment in the apical compartment with control medium or with medium containing 100 ng/ml of LPS (**A**–**C**) or 10 ng/ml of IL1α (**D**–**F**). The concentrations of IL6 (**A**, **C**, **D**, and **F**) were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars) compartments after TER was recorded (**B** and **E**). Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA with Bonferroni post hoc tests. $^{*}P < 0.05$, $^{**}P < 0.01$. ND = below limits of detection.

apically in response to treatment of polarized epithelial cells with bacteria, LPS, or IL1α. The only instances when IL6 accumulated in the basolateral compartment beneath the epithelial cells was when the epithelium was breached by live bacteria or the epithelium was not confluent. We suggest that when faced with infection and damage in the uterine lumen, chemokines attract immune cells to the site of challenge but IL6 is secreted apically when a confluent epithelial cell barrier is present to ensure immune cells are only exposed to IL6 once they reach the uterine lumen (Fig. 8).

The greater accumulation of IL6, but not of CXCL8, in uterine mucus than in peripheral blood in vivo provided an insight that there may be vectorial release of inflammatory mediators. One consideration is that the small variation in the days after parturition when samples were collected might have influenced the concentrations of IL6. However, others also noted higher concentrations of IL6 in uterine fluid than in serum even later postpartum than in the present study [46]. To examine the mechanisms underlying these observations, we used a three-dimensional polarized epithelial cell culture model [24] and examined cellular responses to *E. coli* bacteria and LPS, *T. pyogenes* PLO, and the intracellular cytokine IL1α, which is released by damaged cells [15]. Once a confluent layer of epithelial cells was present as determined by monitoring TER, only IL6 and not CXCL8, CXCL1, CXCL2, CXCL3, and CCL2 was detected solely in the apical, not the basolateral, compartment. Interestingly, the vectorial accumulation of IL6 was independent of whether challenge with LPS was apical, basolateral, or in both compartments. The magnitude of the cellular responses to apical challenge with LPS and IL1α were similar to epithelial cells grown on typical cell culture plates [19]. Also similar to previous studies using LPS, PLO, and IL1α, the challenges did not impact epithelial cell health at the concentrations used in the present study [12,

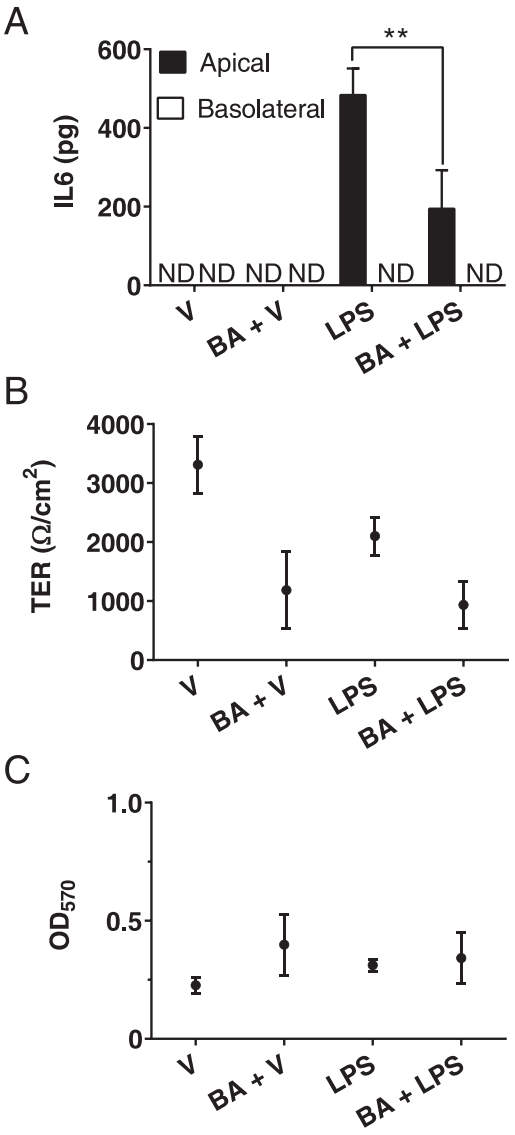


FIG. 6. Trans-Golgi network. Polarized epithelial cells on cell culture inserts were treated for 6 h with control medium or with medium containing 100 ng/ml of Brefeldin A (BA) and for a further 6 h with vehicle (V) or 100 ng/ml of LPS. The concentrations of IL6 (**A**) were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars) compartments after TER was recorded (**B**) and cell viability evaluated by MTT assay (**C**). Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA with Bonferroni post hoc tests. $^{**}P < 0.01$. ND = below limits of detection.

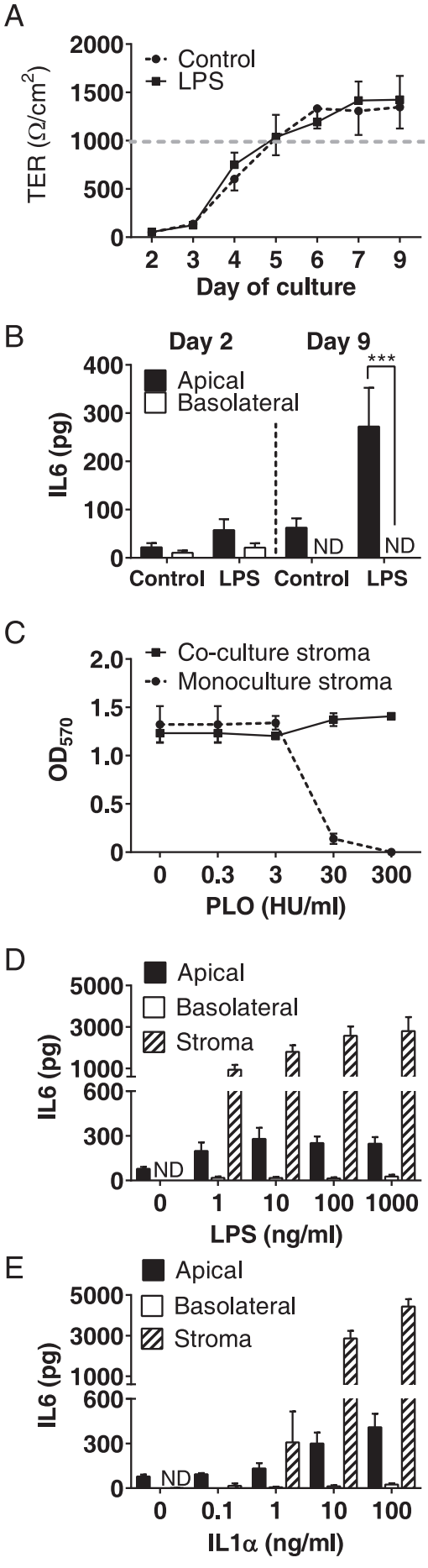
13, 19]. Furthermore, PLO did not stimulate inflammatory responses as expected from previous observations [13].

One concern might be that the present in vitro data were from cells of beef breeds of cattle, whereas the in vivo data were from dairy cows. However, use of different breeds of the same species also provides a wider range of evidence. Furthermore, the epithelial and stromal cell secretion of IL6 or CXCL8 in response to *E. coli* or LPS does not differ significantly between beef and dairy breeds, at least in vitro [38]. In addition, the preferential secretion of inflammatory mediators to the apical surface of polarized epithelial cells is not unusual in other species [1, 2]. In mice, polarized endometrial epithelial cells constitutively accumulate more IL6 apically, although approximately 30% of the total IL6 is found in the basolateral compartment [25]. Similarly, IL6 is principally secreted apically from polarized murine endometrial epithelial cells in response to LPS, but IL6 is also detected basolaterally [27]. Polarized human endometrial epithelial cells also secrete more IL6 apically, although IL6 accumulates in the basolateral compartment constitutively and following treatment with LPS [26]. Polarized epithelial cells from other mucosa, including the cervix, intestine, and respiratory tract also principally secrete IL6 apically, but IL6 is also detectable basolaterally [47–49]. However, the findings in the present study of an exclusive release of IL6 apically and the absence of detectable IL6 in the basolateral compartment appear unique to bovine endometrial epithelial cells. Furthermore, bovine endometrial epithelial cells respond similarly to damage-associated molecules, such as IL1α, whereas polarized murine cells are not responsive to IL1α [25]. Why bovine cells only release IL6 apically is not clear, but this discovery may provide a useful primary cell model for exploring trafficking of proteins in epithelial cells.

The unilateral accumulation of IL6 but not chemokines may reflect their different roles. Chemokines are released to attract migrating hematopoietic immune cells to sites of tissue damage and infection [20]. Thus, for an infection in the uterine lumen, one expects a chemokine concentration gradient from the lumen, across the stroma, toward blood vessels (Fig. 8). The roles of IL6 include enhancing neutrophil recruitment to sites of infection during the early innate immune response, followed later in the course of disease by anti-inflammatory effects, and regulation of the transition from recruiting neutrophils to monocytes [10, 22, 23, 50]. The vectorial IL6 secretion toward the uterine lumen, as found in the present study, may avoid inappropriate activation of immune cells as they migrate across the stroma (Fig. 8). This concept of vectorial release of IL6 to modulate neutrophil function is supported by evidence of apical IL6 secretion from polarized human intestinal epithelial cell lines, which activates neutrophils [48]. If microbes penetrate epithelia and reach the stroma, IL6 would be required in the stroma, and the present data support the notion that the stroma produces abundant IL6 once exposed to pathogens.

Interleukin 6 has a signal sequence that directs delivery into the endoplasmic reticulum, and then to the Golgi, where it is secreted, like most apical proteins, via the trans-Golgi network [2, 40]. In murine macrophages, IL6 and TNF are released via tubulovesicular carriers, which bud off the trans-Golgi network and fuse with recycling endosomes [40]. Consistent with a secretory mechanism employing the trans-Golgi network, in the present study Brefeldin A reduced the accumulation of IL6 in apical cell culture supernatants but did not drive basolateral secretion of IL6. However, unlike macrophages, bovine endometrial epithelial cells do not accumulate detectable concentrations of TNF, whether they are polarized epithelial cells or not [12].

The physiology of the endometrium is influenced by ovarian steroids, and uterine disease is more likely during the luteal than the follicular phase of the estrous cycle [36]. In the present study, exogenous progesterone, estradiol, or a combination of the steroids had little effect on TER or on IL6 secretion, although inflammation was suppressed by dexamethasone as expected [38]. However, caution is required when comparing the in vitro data from the present study with the in vivo situation. For example, the localized concentrations of steroids are higher in the endometrium than in peripheral plasma [51]. Furthermore, culturing cells for several days likely modulates steroid receptor expression and physiological function, although the same model of polarized bovine endometrial epithelial cells do secrete prostaglandins as expected [24]. The present study also supports the recent findings that stage of



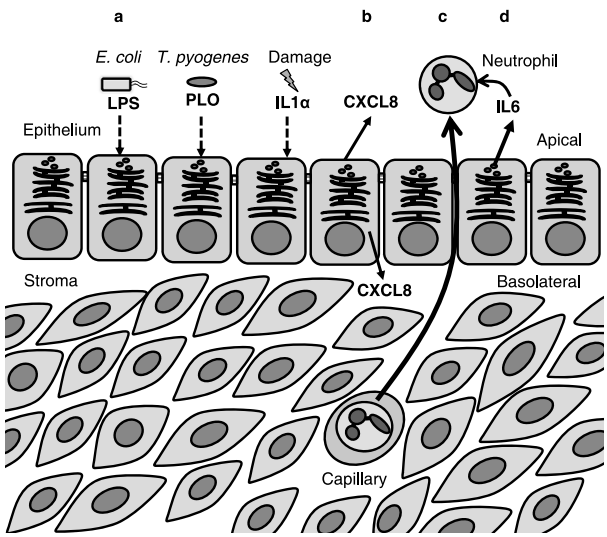


FIG. 8. Roles of polarized endometrial epithelial cells. The polarized endometrial epithelial cells are the first line of defense for the endometrium against ascending infections with gram-negative *E. coli* or gram-positive *T. pyogenes*. **a**) The epithelium protects against bacterial virulence factors, such as LPS and PLO, reaching the underlying stromal cells. **b**) In addition, the epithelial cells sense bacteria and pathogen-associated molecules, such as LPS, and respond to damage-associated molecules, such as IL1α, and secrete CXCL8 and other chemokines, apically and basolaterally. **c**) The chemokine gradient attracts neutrophils from capillaries, and neutrophils migrate across the stroma, through the epithelium, to the site of infection in the uterine lumen. **d**) However, IL6 is only secreted apically from epithelial cells in response to pathogens or tissue damage, and this secretion is via the trans-Golgi network. We suggest that this apical secretion of IL6 avoids inappropriate activation of immune cells by IL6 before they reach the uterine lumen.

estrous cycle, exogenous steroids, or inhibitors for the steroid nuclear receptors did not affect IL6 secretion from conventional bovine endometrial epithelial or stromal cell cultures [38]. In mice, stage of estrous cycle also had little impact on the predominant apical secretion of IL6 from polarized epithelial cells, although exogenous estradiol reduced IL6 release [25].

FIG. 7. Epithelial cells protect the stroma. **A**) Polarized epithelial cells on cell culture inserts were cultured in control medium or with medium containing 100 ng/ml of LPS for up to 9 days, and the TER was recorded daily. **B**) The polarized epithelial cells on cell culture inserts, after 2 or 9 days of culture, were treated in the apical compartment for 24 h with control medium or with medium containing 100 ng/ml of LPS. The concentrations of IL6 were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars) compartments. Data are presented as the mean (SEM) from four independent experiments and were analyzed by ANOVA with Bonferroni post hoc tests. ***$P < 0.001$. ND = below limits of detection. **C**) Confluent polarized epithelial cells on cell culture inserts or cell-free culture inserts were placed above stromal cells in the compartment below, and then the apical compartment was treated with control medium or with medium containing the indicated concentrations of PLO. After 24 h, stromal cell viability was measured using the MTT assay. **D** and **E**) Polarized epithelial cells on cell culture inserts were cocultured with stromal cells in the compartment below, and then the apical compartment was treated with control medium or with medium containing 100 ng/ml of LPS (**D**) or 10 ng/ml of IL1α (**E**), with stromal cells cultured with a cell-free culture insert used as a positive control. After 24 h, the concentrations of IL6 were measured by ELISA in supernatants collected from the apical (solid bars) and basolateral (open bars; often ND) compartments of cocultured cells and from stromal cells (hatched bars). Data are presented as the mean (SEM) additional accumulation of IL6 above the endogenous IL6 secretion measured in the control treatment from four independent experiments.

Similarly, human cells treated with estradiol, but not with progesterone, had reduced IL6 responses to LPS [52].

The protective role of confluent polarized epithelium was evident in the present study as IL6 only accumulated in the basolateral compartment when live *E. coli* penetrated the epithelium to reach the stroma. Furthermore, the polarized epithelium protected underlying cocultured stromal cells from PLO, IL1α, and LPS (Fig. 8). Within a week of parturition, some loss of the epithelium occurs in intercaruncular areas of the endometrium, and the caruncular epithelium has to be regenerated in the period between 1 and 4 wk postpartum [53]. Once the epithelium is breached, this may explain why postpartum infections are readily established and commonly cause endometritis with florid inflammation of the stroma, which leads to infertility [3–5]. The protection afforded by the epithelium against PLO is particularly important as the underlying stromal cells are far more sensitive than epithelial cells to cytolysis caused by PLO [13]. Similarly, far more IL6 is released by stromal than by epithelial cells in response to LPS, bacterial lipopeptides, and IL1α [12, 15]. The greater IL6 response in the stroma might help scale inflammation once the epithelium is breached [54]. An area that warrants further study is the interaction between stroma and epithelium. In mice, stromal cells cocultured with polarized epithelial cells reduced the accumulation of TNF and increased TER [55]. However, in the present study, stromal cells did not influence epithelial IL6 secretion.

In conclusion, the present study showed that polarized bovine endometrial epithelial cells secrete IL6 only apically. The vectorial release of IL6 was independent of whether the cells were challenged apically or basolaterally with pathogen- or damage-associated molecules and was not modulated by hormones. A confluent polarized epithelium was protective against pathogen-associated molecules reaching the underlying stroma, which likely is biologically important during infection of the uterus. In addition, the release of IL6 was dependent on the trans-Golgi network, and the polarized bovine epithelium provides an opportunity for future studies into trafficking of cytokines in mucosa. We suggest that during infection and damage of the uterine lumen, chemokines attract immune cells to the site of challenge but IL6 is secreted apically when a confluent epithelial cell barrier is present to ensure that the immune cells are only exposed to IL6 once they reach the uterine lumen (Fig. 8).

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