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Differential Endometrial Cell Sensitivity to a Cholesterol-Dependent Cytolysin Links *Trueperella pyogenes* to Uterine Disease in Cattle[1]

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ABSTRACT

Purulent disease of the uterus develops in 40% of dairy cows after parturition, when the epithelium of the endometrium is disrupted to expose the underlying stroma to bacteria. The severity of endometrial pathology is associated with isolation of *Trueperella pyogenes*. In the present study, *T. pyogenes* alone caused uterine disease when infused into the uterus of cattle where the endometrial epithelium was disrupted. The bacterium secretes a cholesterol-dependent cytolysin, pyolysin (PLO), and the *plo* gene was identical and the *plo* gene promoter was highly similar amongst 12 clinical isolates of *T. pyogenes*. Bacteria-free filtrates of the *T. pyogenes* cultures caused hemolysis and endometrial cytolysis, and PLO was the main cytolytic agent, because addition of anti-PLO antibody prevented cytolysis. Similarly, a *plo*-deletion *T. pyogenes* mutant did not cause hemolysis or endometrial cytolysis. Endometrial stromal cells were notably more sensitive to PLO-mediated cytolysis than epithelial or immune cells. Stromal cells also contained more cholesterol than epithelial cells, and reducing stromal cell cholesterol content using cyclodextrins protected against PLO. Although *T. pyogenes* or *plo*-deletion *T. pyogenes* stimulated accumulation of inflammatory mediators, such as IL-1beta, IL-6, and IL-8, from endometrium, PLO did not stimulate inflammatory responses by endometrial or hematopoietic cells, or in vitro organ cultures of endometrium. The marked sensitivity of stromal cells to PLO-mediated cytolysis provides an explanation for how *T. pyogenes* acts as an opportunistic pathogen to cause pathology of the endometrium once the protective epithelium is lost after parturition.

bacteria, cholesterol-dependent cytolysin, cow, inflammation, uterus

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INTRODUCTION

Bos taurus dairy cattle are a major source of milk for humans, but productivity is limited by a common endemic disease caused by ubiquitous bacterial infection of the uterus after parturition [1, 2]. Clinical purulent disease of the endometrium is evident in up to 40% of dairy cows, and subclinical disease of the endometrium affects many more animals [1]. Reduced milk production, therapeutics, and replacement of infertile animals cost the combined US and European dairy industries $2 billion annually, as well as compromising food security and animal welfare [1]. After parturition, the single columnar epithelium lining the endometrium of the uterus is denuded, allowing invading bacteria access to the underlying stromal compartment [3–5]. A wide variety of bacteria can be found in the endometrium in the first weeks after parturition, and commonly *Escherichia coli*, *Trueperella pyogenes*, and Gram-positive anaerobic bacteria are isolated from diseased animals [1, 2, 6]. These bacterial infections lead to endometrial tissue damage, infiltration by hematopoietic cells of the immune system, accumulation of pus for a few to several weeks, and increased expression of inflammatory mediators, such as interleukin (IL) -1β, IL-6, and IL-8 [5, 7]. However, among the many bacteria found in the endometrium, Gram-positive *T. pyogenes* is the bacterium most associated with endometritis, and isolation of *T. pyogenes* is correlated with histological and cytological evidence of endometrial pathology [2, 5, 8].

T. pyogenes is commonly isolated from the mucous membranes of ruminants, and is an opportunistic pathogen causing mastitis, abscesses, pneumonia, and endometritis [9]. The primary virulence factor of *T. pyogenes* is the secreted 55-kDa exotoxin, pyolysin (PLO), which is a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins [9]. Typically, CDCs bind cholesterol in plasma membranes and oligomerize to form transmembrane pores 30–50 nm diameter, leading to osmotic imbalance, cell lysis, and death [10]. Similar to a prototypical CDC, such as streptolysin-O (SLO) from *Streptococcus pyogenes*, PLO also binds and forms pore-like structures on cholesterol crystals in a cell-free system [11]. However, PLO is the most divergent CDC family member, and PLO is spontaneously active in vitro, whereas other CDCs, such as SLO, require thiol activation in

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vitro [9]. Although PLO was important for causing pathology in a mouse model of peritonitis [12], it is not clear how *T. pyogenes* causes pathology in the endometrium of postpartum cattle. Endometrial pathogenic *E. coli* is adapted to the bovine endometrium [6], but it is not clear if *T. pyogenes* is also adapted to cause endometrial pathology in cattle. Furthermore, little is known about the interaction of PLO with bovine endometrial cells or how these cells respond to PLO.

There are multiple strategies for defense against microbial damage of tissues, including resistance, tolerance, and immunity [13]. Recognition of microbes by mammalian cells relies on perception of pathogen-associated molecular patterns (PAMPs) [14]. These PAMPs are conserved microbial molecules, such as lipopeptides, found in all bacteria, or the cell wall lipopolysaccharide (LPS) of Gram-negative bacteria. Recognition of PAMPs by hematopoietic cells is mediated by binding to pattern recognition receptors, leading to the production of cytokines, including IL-1β and IL-6, and chemokines, such as IL-8 and other CXCL family members, which attract and activate more hematopoietic immune cells [14]. Although PAMPs, such as bacterial lipopeptides or LPS, play an important synergistic role in the inflammatory response to bacteria that secrete CDCs, it is thought that CDCs may also bind pattern recognition receptors to induce inflammation [15, 16]. Whether PLO stimulates inflammation is not known, but endometrial stromal and epithelial cells express pattern recognition receptors, and these cells mount inflammatory responses to PAMPs, such as LPS [17]. The present study aimed to test the hypothesis that PLO is lytic for host endometrial cells and stimulates an inflammatory response. We found that all clinical isolates of *T. pyogenes* produced PLO, and endometrial stromal cells were more sensitive to PLO-mediated cytolysis than epithelial or immune cells, although none of these cells generated an inflammatory response to PLO.

MATERIALS AND METHODS

Reagents

Reagents were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise specified below. Recombinant PLO (rPLO) [18] and anti-PLO (αPLO) goat antiserum [18] were generous gifts from Prof. B.H. Jost (University of Arizona).

Bacteria

The UK *T. pyogenes* isolates MS249, MS295, MS340, and MS348 (Farm 1), and 683C, 1362B, 50C, and 145B (Farm 2), and Portuguese isolates FMV1 and FMV7 (Farm 3), and FMV13 and FMV23 (Farm 4), were collected previously by endometrial swab from dairy cows with persistent postpartum endometritis, as previously described [2, 19]. Bacteria were identified by aerobic and anaerobic culture on pre-equilibrated sheep blood agar and MacConkey agar (Oxoid, Basingstoke, UK), and identification was based on the characteristics of the colony, Gram stain, morphology, hemolysis, biochemical profile (API systems; BioMérieux, Basingstoke, UK) and other standard tests as described previously [2]. For experiments (see below), bacteria were cultured at 37°C in air using a bacterial growth medium comprised of brain heart infusion (BHI) broth with 5% heat-inactivated fetal bovine serum (FBS; Fisher Scientific, Loughborough, UK), and 0.11% v/v Tween-80. The number of colony-forming units (cfu) was determined by plate counts on sheep blood agar plates. To generate heat-killed *T. pyogenes*, aliquots of bacteria with a known cfu/ml were incubated at 70°C for 15 min and washed three times in ice-cold PBS, followed by centrifugation at 12 000 × *g* for 12 min and resuspended in PBS, with the lack of growth confirmed by attempted culture on sheep blood agar plates for 72 h.

A *plo*-inactivated *T. pyogenes* strain MS249 mutant (*T. pyogenes* Δ*plo*) was generated using a previously reported mutagenesis strategy for *T. pyogenes* [12]. The chromosomal region encoding the *plo* locus was identified in the genome sequence of *T. pyogenes* strain MS249, and primers were designed to amplify a 1148-bp upstream region (PloUF and PloUR) and a 881-bp downstream region (PloDF and PloDR) of the chromosome flanking the *plo* locus, encompassing the first 144 and terminal 249 bases of the 1605-bp *plo* coding region. Primers were also designed to amplify a 981-bp region encompassing the kanamycin resistance cassette and promoter (KanRF and KanRD) from the Epicentre Tn5 Kan-2 transposon (Epicentre, Madison, WI). Restriction enzyme recognition sites listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org) were engineered into the oligonucleotide primers to facilitate cloning of the amplification products, and PCR carried out using Phusion DNA polymerase (NEB, Ipswich, MA) under the following program: 98°C for 3 min, 35 cycles of 98°C 10 sec, 56°C 1 min, 74°C 30 sec, then a single cycle of 74°C for 10 min. The PCR products were digested with appropriate restriction endonucleases (Promega, Madison, WI) according to the manufacturer's instructions. Then, pBluescript SK+ (Stratagene, La Jolla, CA) was digested with *Sal*I/*Eco*RI, and the upstream similarly digested *plo* PCR fragment cloned in. The resulting plasmid was then digested with *Eco*RI/*Xba*I and the downstream similarly digested *plo* PCR product was cloned in. This plasmid, which houses the upstream and downstream *plo* flanking regions separated by an *Eco*RI restriction site, was then digested with *Eco*RI, and the similarly digested kanamycin resistance cassette PCR product inserted to yield the plasmid pBSplo_Kan in which the *plo* coding sequence is interrupted and replaced by kan-2. As the plasmid pBluescript SK+ is based on a ColE replicon, and cannot replicate within *T. pyogenes*, it acts as a suicide vector, which has been used previously for mutagenesis in this organism [12]. The *T. pyogenes* MS249 cells were transformed with pBSplo_Kan by electroporation, as described previously [20]. Transformants were selected by growth on BHI agar with 10% FBS supplemented with 30 μg/ml kanamycin and subsequently plated onto sheep blood agar plates (Oxoid) to confirm the absence of PLO-mediated hemolysis [12].

Bovine Cell and In Vitro Organ Cultures

Uteri and blood were collected from animals after slaughter, using animals that were processed as part of the normal work of an abattoir. In vitro organ cultures and isolation and culture of endometrial epithelial cells and stromal cells were as described previously [17, 21, 22]. Briefly, bovine uteri were collected from postpubertal, nonpregnant animals with no evidence of genital disease or microbial infection, and the uteri were kept on ice until further processing in the laboratory. Postpartum cattle were not used, because experiments would be confounded by the already existent ubiquitous bacterial contamination and inflammation of the endometrium, disruption of the epithelium, and the presence of tissue damage [1, 3, 4]. The stage of the reproductive cycle was determined by observation of ovarian morphology, as described previously [23]. Genital tracts were selected for endometrial culture from animals on Days 1–4 of estrous cycle when an ovary contained a recently ruptured follicle (Ovarian Stage I) [23]; similar to postpartum cows, these animals have basal peripheral plasma progesterone concentrations [2, 17].

For endometrial cell culture, endometrium from the horn ipsilateral to the recently ruptured follicle was dissected and processed as described previously [17, 21]. Tissue was digested in 25 ml sterile digestive solution comprised of 375 BAEE units trypsin ethylenediaminetetra-acetic acid (EDTA), 50 mg collagenase II, 100 mg bovine serum albumin (BSA) and 10 mg DNase I in 100 ml Hanks balanced salt solution (HBSS) for 1 h at 37°C with gentle agitation. The cell suspension was filtered through a 40-μm mesh (Fisher Scientific) to remove undigested material and the filtrate was resuspended in wash medium comprised of 10% FBS (Biosera, Ringmer, UK) in HBSS. The suspension was washed three times by centrifuging at 100 × *g* for 10 min and resuspending in wash medium. Finally, the cells were resuspended in culture medium comprised of RPMI-1640 medium, 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, and plated in 75-cm^2 tissue culture flasks (Greiner Bio-One, Stonehouse, UK). To obtain separate stromal and epithelial cell populations, the cells were treated with Accutase until the stroma had detached; these were then replated. Stromal and epithelial cell populations were determined to be >99% pure as described previously [17]. The culture media were changed every 48 h until the cells reached approximately 70% confluence, at which point they were transferred into 24-well tissue culture plates (TPP, Trasadingen, Switzerland), or Cellview glass chamber slides (Greiner Bio-One) for treatment. Cell cultures were maintained at 37°C, 5% CO_2, in a humidified incubator and treatments applied within 10 days (see below). At the end of the treatment period, the culture supernatants were harvested and stored at −20°C and cell survival assessed.

Cell survival was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, as described previously [24]. Briefly, for the MTT assay, once the supernatants were removed, they were replaced with fresh culture medium containing 0.5 mg/ml MTT and incubated with the cells for 1 h; the medium was then removed and the cells washed with Dulbecco PBS (DPBS) before

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lysis with dimethyl sulfoxide, and measurement of optical density (OD) at 570 nm using a microplate reader (POLARstar Omega; BMG Labtech, Offenburg, Germany). The correlation between MTT OD measurements and the number of live cells was confirmed using trypan blue exclusion and counting the number of live cells using a hemocytometer.

For in vitro organ cultures, intact endometrium was collected from the intercaruncular areas of the endometrium using sterile 8-mm-diameter biopsy punches (Stiefel Laboratories Ltd, NC), and the endometrium was dissected from the underlying tissue using sterile scissors and processed as described previously [22]. Briefly, each sample of endometrium was immediately transferred to HBSS supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B for 5 min. The samples were then washed twice in unsupplemented HBSS and transferred to six-well tissue culture plates. Each well contained a single organ culture in 3 ml of culture medium. The organ cultures were orientated with the epithelial surface uppermost, and treatments applied (see below) for 24 h in a humidified atmosphere with 5% CO_2 in air at 37°C. The supernatants were then harvested and frozen at −20°C until required for further analysis, and each organ culture was weighed so that the production of inflammatory mediators could be normalized to the organ weight.

Peripheral blood mononuclear cells (PBMCs) were isolated and cultured as described previously [25]. Blood was collected from cattle within 5 min of slaughter into tubes containing 1 mg/ml EDTA and 50 μg/ml gentamicin for 1 h at room temperature. The blood was added to an equal volume of HBSS containing gentamicin and amphotericin B. A total of 16 ml of the mixture was then carefully added to 12 ml Ficoll-Paque Premium (GE Healthcare, Chalfont St. Giles, UK) before centrifugation at $500 \times g$, 18°C, for 40 min with the brake off. The PBMCs were harvested from the dense, white layer between the resulting Ficoll-Paque and plasma layers. The cells were carefully transferred to a 15-ml Falcon tube, resuspended with a Pasteur pipette, and washed twice by adding 15 ml supplemented HBSS before being centrifuged for 15 min. The cells were then resuspended in 5 ml RPMI and added to a 60-mm Petri dish (Greiner Bio-One) and incubated at 37°C, 5% CO_2, for 30 min to allow the PBMCs to adhere. The media were then removed and the dishes washed twice with HBSS before 5 ml culture medium was added. The cells were cultured at 37°C, 5% CO_2, for 6–8 days with media changes every 48 h. When required for treatment, the cells were transferred into 24-well tissue culture plates and treated within 24 h of transfer.

In Vivo Bacterial Challenge Model of Endometritis

To confirm that *T. pyogenes* is a primary uterine pathogen, an in vivo bacterial challenge model of bovine endometritis was used, based on a previously described model [26]. The Kalamazoo Institutional Animal Care and Use Committee approved the animal procedures (Animal Use Protocol number KZ-1599e-2011-06-jes), under the Animal Welfare Act of 1966 as administered by the Animal and Plant Health Inspection Service of the United States Department of Agriculture. Lactating Holstein dairy cows (range, two to four lactations), 205 days postpartum, with no uterine disease and no antibiotic treatment within 14 days of enrolment, were randomly assigned to intrauterine infusion of 10 ml bacterial growth medium containing 5×10^8 cfu of a uterine isolate of *T. pyogenes* ($n = 5$), or a no-treatment control group ($n = 5$). The perineum was disinfected with chlorhexidine; the endometrium scarified using a sterile cytobrush instrument inserted through the cervix, guided by palpitation per rectum; and the bacterial suspension infused using a sterile uterine infusion cannula. Vaginal discharge was examined daily to evaluate uterine disease, and uterine swabs collected on Days 0, 2, 5, 7, 9, 14, 21, and 28 after challenge for bacterial culture, as described previously [2]. The control group was used to determine if animals remained free of *T. pyogenes* and uterine disease, despite undergoing the same invasive monitoring procedures.

Bacterial Genomic Sequences for PLO

The 12 *T. pyogenes* isolates from the endometrium of animals with purulent uterine infections were examined for their DNA sequence of the *plo* gene and gene promoter and compared with the previously published nucleotide sequence for *plo* (GenBank accession no. U84782.2). The DNA sequences were retrieved from genome sequences of the *T. pyogenes* isolates MS249, MS295, MS340, and MS348; and by PCR for the remaining eight isolates (GenBank accession nos. KJ150322–KJ150329) using the primers PloUF (GTGTCGACCCTCCTGTTCAG), PloUR (GCCGAATTCAGGTCAACTT TATC), PloDF (AAGGAATTCCGTACACCCAAG), PloDR (TCGTCTAGA TACGCAGAATCATGAG), PloF (GTGTGCGCTCAATACGCTAT), and PloR (TTCACAACTGTTCGCCACTG), to generate three overlapping PCR products, which were sequenced by dideoxy chain termination/cycle sequencing on an ABI 3730XL sequencing machine (Eurofins MWG Operon, Ebersberg, Germany), and assembled in silico to produce a sequence covering the *plo* locus and flanking regions. Protein sequences for PLO present in the National Center for Biotechnology Information database (Genbank accession nos. AAC45754, ADU04496, BAA86937) were collected along with the *Arcanobacterium hemolyticum* DSM 20595 thiol-activated cytolysin sequence (YP_003697395) and the *Streptococcus suis* thiol-activated cytolysin sequence (YP_006358469). Sequences were used in phylogeny.fr webserver to build a phylogenetic tree as described previously [27].

Bacterial Growth Curves

To explore how different clinical isolates of *T. pyogenes* behave, standard bacterial growth curves were measured for each of the 12 uterine isolates of *T. pyogenes*. Briefly, 50 ml bacterial growth medium (BHI broth with 5% heat-inactivated FBS and 0.11% v/v Tween-80) was inoculated with a starting OD_{600} of 0.002, cultured at 37°C in air, and OD_{600} measured by spectrophotometer (BioQuest, South San Francisco, CA) every 2 h for 24 h on three independent occasions for each isolate. In addition, bacteria-free filtrates (BFF) were made by collecting each of the *T. pyogenes* growth supernatants every 2 h, followed by centrifugation at $3200 \times g$ for 10 min, and then passing the remaining supernatant through a low-binding 0.22-μm syringe filter (Millipore, Billerica, MA).

Hemolytic Activity

CDCs cause hemolysis of red blood cells, and a standard hemolysis assay was employed as a functional measure of cytolytic activity, using a previously reported method [18, 28]. The hemolytic activity of BFF was measured every 2 h during the 24-h bacterial growth curve experiment for each of the 12 *T. pyogenes* isolates using triplicate replicates for each isolate. The hemolysis assay was also used to measure the potency of rPLO and SLO cytolysin proteins. Briefly, one hemolytic unit (HU) was defined as the amount of BFF or cytolysin releasing 50% of the hemoglobin from red blood cells after incubation at 37°C for 1 h. The BFF or cytolysin samples were serially diluted 1:2 across a round-bottomed 96-well plate (Fisher Scientific) in DPBS with 1% v/v BSA (DPBS/BSA). The negative control was DPBS/BSA solution and the positive control was 1% v/v Triton X-100 in DPBS/BSA. To prepare the washed red blood cells, commercially available defibrinated horse blood (Oxoid, Basingstoke, UK) was centrifuged at $1500 \times g$ for 10 min, 4°C. The supernatant was removed and the pelleted red blood cells were resuspended in DPBS/BSA. The process was then repeated three to four times until the supernatant was colorless. Subsequently, 500 μl of the red blood cell pellet was added to 100 ml DPBS/BSA to make a 0.5% stock solution of washed red blood cells; 100 μl washed red blood cells were added to each 100-μl sample or control in the plate, giving a final concentration of 0.25% red blood cells. The samples were incubated at 37°C for 1 h before centrifuging at $1500 \times g$ for 5 min at room temperature to pellet intact red blood cells. Finally, 100 μl of the resulting supernatant was transferred into a flat-bottomed 96-well plate (Fisher Scientific) before the OD_{405} of the supernatant was measured in a plate reader (PolarStar; BMG, Ortenberg, Germany). The point at which 50% of the hemoglobin was released from the red blood cells was calculated graphically using the solver function of Microsoft Excel (Microsoft Corporation), setting the positive and negative controls as the maximum and minimum limits, respectively.

PLO in BFF

A previously reported Western blot method was used to confirm that PLO protein was present in the BFF collected from the growth of *T. pyogenes* [18]. The BFF was extensively dialyzed (12- to 14-kDa cutoff membrane; Medicell, London, UK) against PBS and concentrated 10-fold in a protein concentrator (Eppendorf, Stevenage, UK). A total of 25 μl of BFF was then separated on a 12% polyacrylamide gel (mini-protean TGX; Bio-Rad, Hemel Hempstead, UK), transferred to a PVDF membrane (GE Healthcare), and incubated in blocking buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 5% w/v BSA, and 0.1% v/v Tween-20) overnight at 4°C. Membranes were probed with 1:100 dilution of αPLO goat serum [18] in blocking buffer for 2 h and washed for 5 min three times in wash buffer (20 mM Tris, pH 7.6, 125 mM NaCl, and 0.1% v/v Tween-20). A 1:1000 dilution of anti-goat IgG-HRP (Cell Signaling Technology, Danvers, MA) was added for 1 h at room temperature before the wash step was repeated. Immunoreactive bands were visualized using Luminata Crescendo Western HRP substrate (Millipore), and images collected using a Chemi-Doc imaging system (Bio-Rad).

Cellular Sensitivity to PLO

To explore the impact of PLO, red blood cells, endometrial epithelial cells, and stromal cells were treated for 1 h with control culture medium or medium

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containing a range of concentrations of BFF from *T. pyogenes* isolate MS249 (10–500 HU as indicated in *Results*). Red blood cell and endometrial cell survival was monitored by hemolysis assay and MTT assay, respectively, and data were expressed as a percentage of the control. The experiment was repeated on three independent occasions. To evaluate the cellular sensitivity to each of the 12 clinical isolates, the BFF of each isolate was diluted to 150 HU in culture medium before treating endometrial epithelial and stromal cells for 1 h; cell survival was monitored by MTT assay, and data were expressed as a percentage of the control. The experiment was repeated on three independent occasions.

To test if cytolysis by BFF was specifically caused by native PLO in BFF, red blood cells and endometrial stromal cells were treated with 150 HU BFF for 1 h with a blocking antibody for PLO (αPLO) [18]; cell survival was monitored by hemolysis assay and MTT assay, respectively, and data were expressed as a percentage of the control. The experiment was repeated on three independent occasions.

To specifically explore the impact of PLO, endometrial stromal and epithelial cells were treated for 24 h with control culture medium or medium containing rPLO [18] using a range of concentrations (0.3–30 HU, as specified in *Results*); cell survival was monitored by MTT assay, and data were expressed as a percentage of the control. The experiment was repeated on four independent occasions. To verify the changes in MTT OD observed for stromal cells, these cells were also cultured on glass chamber slides (CELLview, Greiner Bio-One) and treated with 3 or 30 HU rPLO for 24 h before staining with Hoechst 33342 to visualize cell nuclei using an epifluorescent microscope (Axio Imager.M1; Zeiss, Jena, Germany). The experiment was repeated on three independent occasions.

To evaluate the impact of native PLO secreted by *T. pyogenes*, endometrial cells were treated with control culture medium or medium containing live *T. pyogenes* (strain MS249) or the same strain of *T. pyogenes* that lacked functional *plo* (*T. pyogenes* Δ*plo*). Cells were treated for 24 h with control culture medium or medium containing a range of multiplicities of infection (MOIs) of MS249 (0.01 to 100 MOI, as indicated in *Results*) or 100 MOI *T. pyogenes* Δ*plo*. In independent experiments, red blood cells or endometrial epithelial and stromal cells were treated with control culture medium or medium containing 1, 10, or 100 MOI *T. pyogenes* Δ*plo*. Cell survival was monitored by hemolysis assay or MTT assay, respectively, and data were expressed as a percentage of the control. The experiments were repeated on four independent occasions.

To test if endometrial cell sensitivity was generalized for other members of the CDC family, endometrial cells were treated for 24 h with control culture medium or medium containing 0.003–30 HU of rPLO, or 0.003–30 HU 10 mM DL-Dithiothreitol (DTT)-activated SLO from *S. pyogenes*, which is not a pathogen of the bovine uterus [2]. In addition, cells were also treated with 10 mM DTT to evaluate DTT toxicity, and with 1 μg/ml ultrapure LPS from *E. coli* O111:B4 (Invivogen, Toulouse, France) to examine whether the prototypical PAMP might impact cell survival. Cell survival was monitored by MTT assay, and data were expressed as a percentage of the control. The experiment was repeated on four independent occasions.

Hematopoietic immune cells are important in endometrial pathology, and so neutrophils, monocytes, and lymphocytes were examined for sensitivity to rPLO. Briefly, 40-ml samples of venous blood were collected into heparin-containing tubes (BD, Heidelberg, Germany) from three Holstein cows, and the cells labeled with R-phycoerythrin-conjugated anti-CD14 monoclonal antibody (AbD Serotec, Oxford, UK) for 30 min at 4°C in PBS containing 0.5% BSA and 0.01% sodium fluoride. After washing twice in PBS, 3×10^5 cells were seeded in round-bottomed, 96-well plates and incubated in control culture medium or medium containing 0.003–100 HU/well PLO (as specified in *Results*) for 1 h at 37°C, with 5% CO_2 in air. The number of viable neutrophils, monocytes, and lymphocytes were quantified by flow cytometry using an Accuri cytometer (BD) and at least 30 000 events, as described previously [29].

The Kinetics of Cellular Sensitivity to PLO

The kinetics of PLO activity were explored using fluorescent-labeled phalloidin, which is normally excluded from cells with an intact plasma membrane, but can gain entry to the cytosol through CDC pores, where phalloidin binds to F-actin and fluoresces as described previously [30]. Briefly, endometrial stromal and epithelial cells were seeded in four-chamber CELL-view chamber slides. After 16 h, Hoechst 33342 (Invitrogen, Paisley, UK) diluted 1:1000 in culture medium was added, and images collected before addition of 30 HU rPLO along with Alexa Fluor 555 phalloidin (Invitrogen) to give a final dilution of 1:100. Samples of three independent experiments were analyzed on an LSM710 confocal microscope (Zeiss), using the diode (405 nm excitation) and helium-neon (543 nm) lasers, and a 20× C-apochromat objective. Images were collected and cellular florescence intensity measured by confocal microscopy every 3 min, using the Zen 2009 software (Zeiss).

Cellular Cholesterol and Sensitivity to PLO

PLO is a member of the family of CDCs, which bind cholesterol-rich areas of the plasma membrane of cells to form pores, and can bind and form pore-like structures on cholesterol crystals in a cell-free system [9, 11]. As cholesterol is essential for the action of CDCs, bovine endometrial epithelial and stromal cell cholesterol content was measured on three independent occasions using the Amplex Red Cholesterol Assay Kit (Invitrogen), according to the manufacturer's instructions.

Cyclodextrins were used to further explore the role of cellular cholesterol, because they deplete cholesterol from plasma membranes [31]. Cyclodextrins, and particularly methyl-β-cyclodextrin, bind and sequester cholesterol molecules, and the consequent removal of cholesterol from cell membranes can reduce the hemolytic activity of CDCs [10, 31]. To evaluate the impact of cyclodextrins on stromal cell cholesterol content, endometrial stromal cells were treated for 0 and 24 h with 1000 μM α-cyclodextrin, methyl-β-cyclodextrin, or γ-cyclodextrin before measuring cellular cholesterol concentration, on three independent occasions. To evaluate if depletion of cellular cholesterol protected stromal cells against PLO-mediated cytolysis, cells were treated with control culture medium or medium containing a range of concentrations for each of α-cyclodextrin, methyl-β-cyclodextrin, or γ-cyclodextrin (1, 10, 100, and 1000 μM) for 24 h before treatment with control culture medium or medium containing 15 HU rPLO for 24 h; cell survival was monitored by MTT assay, and data were expressed as a percentage of the control. The experiment was repeated on four independent occasions for each cyclodextrin.

Cellular Responses to *T. pyogenes*

As endometrial pathology involves inflammation as well as tissue damage, the cellular inflammatory responses to *T. pyogenes* and PLO were explored using in vitro organ cultures, endometrial cells, and hematopoietic immune cells. In vitro organ cultures were treated for 24 h with control culture medium, or medium containing live *T. pyogenes* (strain MS249; 10^3–10^7 cfu/ml), the same strain of *T. pyogenes* that lacked functional *plo* (*T. pyogenes* Δ*plo*; 10^3–10^7 cfu/ml), or rPLO (0.003–30 HU), or the prototypical PAMP LPS as a positive control using 1 μg/ml ultrapure LPS, as described previously [17, 32]. Experiments were repeated on four independent occasions, with treatments applied to duplicate replicate wells in 24-well cell culture plates. Supernatants were collected and stored at −20°C until being used for ELISA to measure IL-1β, IL-6, and IL-8.

Endometrial epithelial and stromal cells were treated for 24 h with control culture medium, medium containing heat-killed *T. pyogenes* MS249 (equivalent of 0.1–1000 MOI, as specified in *Results*), or rPLO (0.003–30 HU, as specified in *Results*), or 1 μg/ml ultrapure LPS as a positive control. To examine if cellular responses to PLO were indicative of other CDCs, stromal cells were also treated with 0.003–30 HU SLO activated using 10 mM DTT, or 1 μg/ml ultrapure LPS as a positive control PAMP. Experiments were repeated on four independent occasions, with treatments applied to duplicate replicate wells in 24-well plates. Supernatants were collected and stored at −20°C until being used for ELISA to measure IL-1β, IL-6, IL-8, CXCL1, CXCL2, and CXCL3.

To examine if hematopoietic cells generate inflammatory responses to PLO, PBMCs were treated for 24 h with control culture medium or medium containing rPLO (0.003–3 HU, as specified in *Results*), or 1 μg/ml ultrapure LPS as a positive control. Experiments were repeated on four independent occasions, with treatments applied to duplicate replicate wells in 24-well plates. In addition, PBMC survival was monitored by MTT assay, and data were expressed as a percentage of the control.

ELISA

Concentrations of IL-1β, IL-6, and IL-8 were measured in culture supernatants by ELISA according to the manufacturers' instructions (Bovine IL-1β Screening Set ESS0027 and Bovine IL-6 Screening Set ESS0029 [Thermo Fisher Scientific]; Human CXCL8/IL-8 DuoSet DY208 [R&D Systems, Abingdon, UK]). The Human CXCL8/IL-8 DuoSet was previously validated for measurement of bovine IL-8 [33]. The limits of detection for IL-1β, IL-6, and IL-8 were 20.1, 35.6, and 14.3 pg/ml, respectively; the intra-assay coefficients of variance were 4.6%, 1.2%, and 1.7%, respectively, and the interassay coefficients of variance were 7.7%, 3.0%, and 5.5%, respectively. Concentrations of CXCL1, CXCL2, and CXCL3 were measured by ELISA, as described previously [34]; the limits of detection were <60, 300, and 400 pg/ml, respectively, and inter- and intra-assay coefficients of variation were all <8% and <5%, respectively [34]. Data are reported as pg/mg of tissue for organ culture, and pg/ml for cell culture experiments.

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Statistical Analysis

Data are presented as arithmetic mean (SEM). Statistical analyses used *t*-test, or ANOVA with Bonferroni or Dunnett pairwise multiple comparison *t*-test (SPSS ver. 16; SPSS Inc., Chicago, IL) to compare means. Comparison of proportions was tested by Chi-square test, and correlations were examined using Pearson or Kappa tests. Significance was ascribed at $P < 0.05$.

RESULTS

T. pyogenes *as a Primary Pathogen*

To establish whether *T. pyogenes* acted as a primary uterine pathogen, an in vivo bacterial challenge model of bovine endometritis was used, based on a previously described model [26]. Holstein cows with no history of uterine disease were randomly assigned to intrauterine infusion of *T. pyogenes* or untreated control. Mucopurulent uterine discharge was evident in four out of five infused animals and one out of five control animal, and *T. pyogenes* was consistently isolated from animals infused with bacteria (five out of five), but not control animals (zero out of five). Mucopurulent discharge was associated with isolation of *T. pyogenes* on Days 4 and 6 after challenge (Kappa 0.35 and 0.36, respectively; $P < 0.05$), and discharge persisted for 18 ± 2 days in infused animals.

T. pyogenes *Expression of PLO*

The *plo* gene and *plo* gene promoter was sequenced for 12 *T. pyogenes* isolates from the endometrium of animals with purulent uterine infections; eight isolates were from two UK farms [2], and four isolates were from two Portuguese farms [19]. The *plo* gene was identical among the 12 isolates (Supplemental Fig. S1). Furthermore, the *plo* gene of the 12 isolates was identical to a previously published sequence (GenBank accession no. U84782.2). The nucleotide sequence of the *plo* promoter was also highly conserved, with only minor variations among the 12 strains of *T. pyogenes* (Supplemental Fig. S2). The 12 isolates also had highly similar bacterial growth characteristics (Supplemental Fig. S3A). The bacterium is hemolytic, and the kinetics of hemolytic activity was similar across the growth curve for the 12 isolates, with maximal hemolytic activity in early stationary phase of growth (Supplemental Fig. S3B). The BFF from cultures of each of the 12 bacterial isolates caused hemolysis, as determined using the standard hemolysis assay (Supplemental Fig. S3C), and PLO protein was identified in BFF by Western blot (Supplemental Fig. S3D).

To test if PLO in BFF was functional, BFF from *T. pyogenes* isolate MS249 (10–500 HU) was added to red blood cells or endometrial stromal and epithelial cells for 1 h, before assessment of cell survival by hemolysis or MTT assays, respectively. The MTT assay does not rely on dye exclusion or plasma membrane integrity, and has been used previously to measure cell viability in response to CDCs [35]. There was also a linear relationship between MTT OD and the number of viable endometrial cells ($r^2 = 0.99$, $P < 0.001$, Pearson correlation). The lowest concentration of BFF, equivalent to 10 HU in the hemolysis assay, caused complete lysis of red blood cells (Fig. 1A). Stromal cell lysis was evident with ≥50 HU of BFF, and few cells survived ≥250 HU of BFF, whereas epithelial cell survival was only significantly reduced by 500 HU BFF (Fig. 1A). The BFF from each *T. pyogenes* isolate, adjusted to 150 HU, also caused more stromal than epithelial cytolysis (26 ± 3% vs. 83 ± 9% survival compared with control culture medium, $P < 0.05$, ANOVA), with no significant difference in potency among isolates (Fig. 1B). To test if cytolysis was specifically caused by native PLO in BFF,

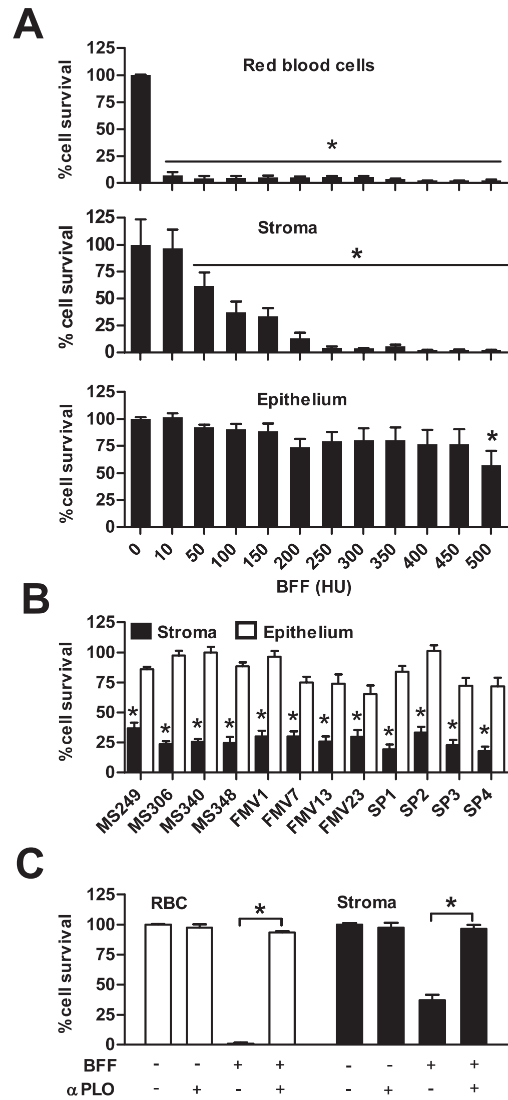

FIG. 1. Cytolytic activity of *T. pyogenes* BFF. **A**) Cytolysis of red blood cells and bovine endometrial stromal and epithelial cells treated for 1 h with the indicated concentrations of BFF, as determined by hemolysis assay for red blood cells, and MTT assay for endometrial cells. Data are expressed as a percent of control, and values differ from control by ANOVA and Dunnett pairwise *t*-test; *$P < 0.05$. **B**) Survival of endometrial stromal and epithelial cells treated for 1 h with 150 HU BFF from each of 12 *T. pyogenes* isolates. Data are expressed as a percent of control, and differences between cell type were evaluated by independent *t*-test; *$P < 0.05$. **C**) Survival of red blood cells and stromal cells when treated with control culture medium or medium containing 150 HU BFF *T. pyogenes*, with or without anti-PLO antibody (αPLO) for 1 h. Data are expressed as a percent of control, and values differ where indicated, as determined by ANOVA and Bonferroni post hoc test; *$P < 0.05$. Data are presented as mean (SEM) of cells from 3 animals for each experiment.

red blood cells and stromal cells were treated with 150 HU BFF for 1 h with a blocking antibody for PLO (αPLO) [18], and this approach prevented both hemolysis and endometrial stromal cytolysis (Fig. 1C).

Differential Cell Sensitivity to PLO

To explore the impact of PLO, endometrial cells were treated for 24 h with rPLO [18]. Fewer stromal than epithelial cells survived treatment with >20 HU rPLO (19.8 ± 1.7 vs.

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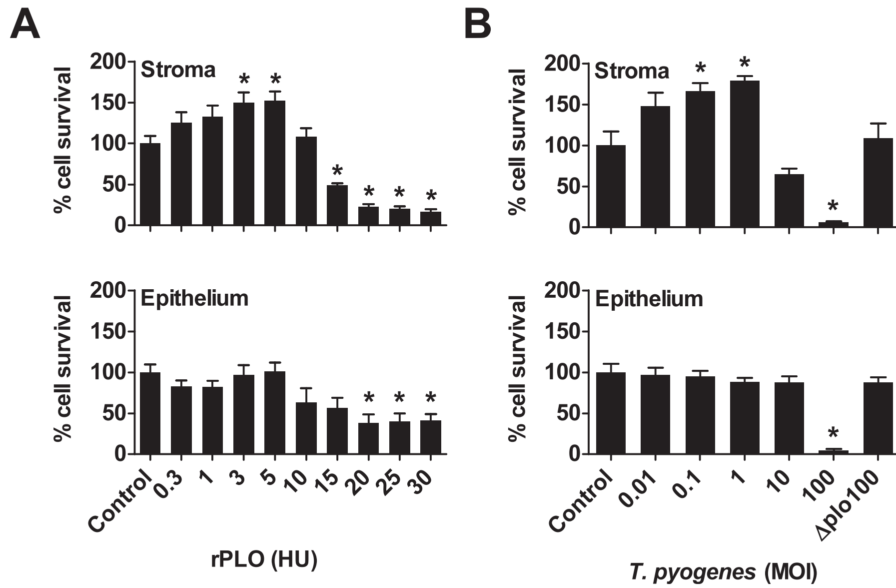

FIG. 2. Cytolytic activity of PLO and *T. pyogenes*. Percentage survival of stromal and epithelial cells after 24-h treatment with the indicted concentrations of rPLO (**A**) or the indicated MOI of live *T. pyogenes* (**B**), or 100 MOI of the same strain that lacked functional *plo* (Δ*plo*). Data are expressed as a percent of control, and are presented as mean (SEM) of four independent experiments. Values differ from control by ANOVA and Dunnett pairwise *t*-test; *$P < 0.05$.

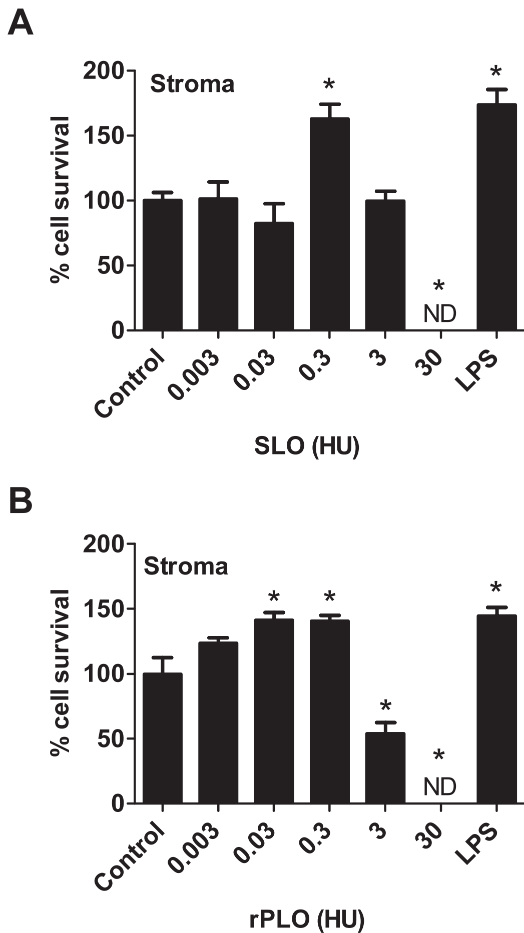

FIG. 3. Stromal cell survival after treatment with CDCs. Stromal cells were treated with 0.003–30 HU of SLO (**A**) or rPLO (**B**) for 24 h, and 1 μg/ml LPS as a positive control. Cell survival was assessed by MTT assay. Data are presented as mean (SEM) of four independent experiments. Differences from control were assessed by ANOVA and Dunnett two-sided comparison; *$P < 0.05$. ND, values below the limits of detection.

40.0 ± 5.0% survival, $P < 0.001$, ANOVA), although sublytic concentrations of <10 HU rPLO increased the number of stromal cells compared with control (Fig. 2A). To verify the changes in MTT OD, stromal cells were cultured on glass chamber slides and treated with 3 or 30 HU rPLO for 24 h before staining with Hoechst 33342 to visualize cell nuclei. There were more Hoechst 33342-positive nuclei when stromal cells were treated with 3 HU rPLO than control culture medium (219 ± 35 vs. 128 ± 15 cells/mm^2, $P < 0.05$, *t*-test), whereas few stromal cells survived treatment with 30 HU rPLO (5 ± 1 vs. 128 ± 15 cells/mm^2, $P < 0.05$, *t*-test).

To evaluate the impact of native PLO, endometrial cells were treated with a range of MOIs of live *T. pyogenes* strain MS249. An MOI of 100 *T. pyogenes* reduced stromal and epithelial cell survival after 24 h (Fig. 2B). However, there was no detectable cytolysis when stromal cells were treated with 100 MOI of the same strain of *T. pyogenes* that lacked functional *plo* (*T. pyogenes* Δ*plo*; Fig. 2B). Furthermore, the BFF from *T. pyogenes* Δ*plo* bacteria did not cause hemolysis (HU = 0). An increase in the number of stromal, but not epithelial, cells was evident following treatment with <10 MOI live *T. pyogenes* (Fig. 2B). However, 1, 10, or 100 MOI *T. pyogenes* Δ*plo* did not significantly impact the number of stromal or epithelial cells (Supplemental Fig. S4, A and B).

To test if differential endometrial cell sensitivity was generalized for other members of the CDC family, endometrial cells were treated with SLO from *S. pyogenes*, which is not a pathogen of the bovine uterus [2]. Following DTT activation, which is necessary for most CDCs apart from PLO, 30 HU SLO caused 100% stromal cytolysis after 24 h, although 3 HU did not significantly affect cell survival (Fig. 3A). This is in contrast to 3 HU rPLO within the same experiment, which reduced stromal cell survival by 53.9 ± 8.9% of control (Fig. 3B). Data for epithelial cells were not obtained, because DTT was toxic and DTT alone caused rapid epithelial, but not stromal, cell death.

Hematopoietic immune cells are also important in endometrial pathology, and so neutrophils, monocytes, and lymphocytes were treated with a range of concentrations of rPLO for 1

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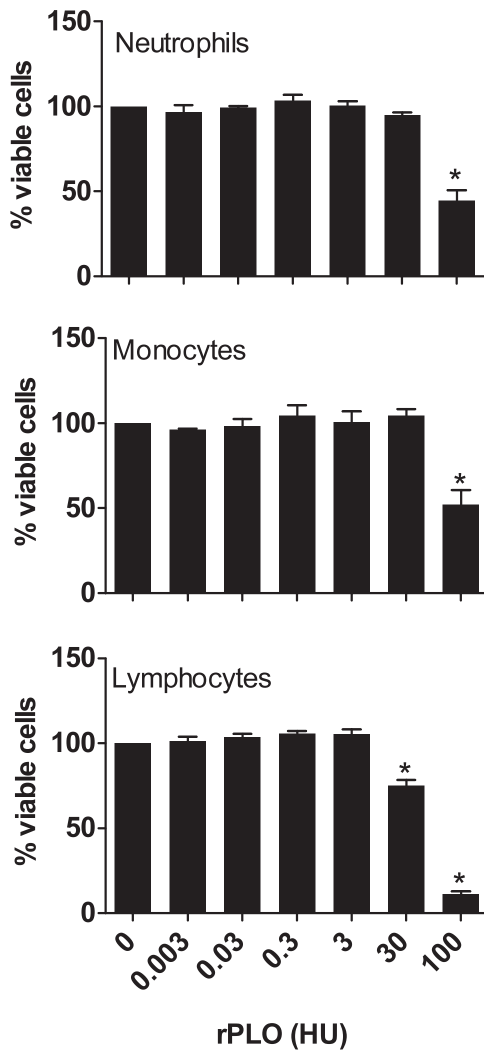

FIG. 4. Hematopoietic immune cell survival after treatment with PLO. Neutrophils, monocytes, and lymphocytes were isolated from bovine blood and treated with 0.003–100 HU rPLO for 1 h before FACS analysis of cell survival. Data are presented as mean (SEM) of three independent experiments. Values differ significantly by ANOVA and Dunnett two-sided post hoc comparison with control (0); $^*P < 0.05$.

h to examine cytolysis. Addition of 100 HU rPLO reduced neutrophil, monocyte, and lymphocyte survival to 44.7 ± 6.0%, 52.1 ± 8.6%, and 11.2 ± 1.8% of cells in control media, respectively (Fig. 4). However, 3 HU rPLO did not significantly affect neutrophil, monocyte, or lymphocyte survival.

The Kinetics of PLO Action

The kinetics of PLO activity were explored using fluorescent-labeled phalloidin, which is normally excluded from cells with an intact plasma membrane, but can gain entry to the cytosol through CDC pores, where phalloidin binds to F-actin and fluoresces [30]. Endometrial stromal and epithelial cells were concurrently treated with rPLO and fluorescent-labeled phalloidin, and fluorescence monitored by confocal microscopy. After 2 h, 57 ± 1% of stromal cells and, after 4 h, 94 ± 4% of stromal cells had phalloidin ingress (Fig. 5A), whereas fewer epithelial cells were fluorescent even after 4 h (15 ± 1%, $P < 0.001$, Chi-square test; Fig. 5B). In addition, the fluorescence intensity associated with phalloidin ingress was higher for stromal than epithelial cells (128 ± 4 arbitrary units/cell [n = 164] vs. 81 ± 5 arbitrary units/cell [n = 56], $P < 0.001$, *t*- test). The rate of increase in fluorescence intensity was more rapid for cells treated with rPLO than in control culture medium (Fig. 5, C and D; $P < 0.001$, ANOVA), and more rapid for stromal than epithelial cells treated with rPLO ($P < 0.001$, ANOVA).

Cellular Cholesterol and Sensitivity to PLO

PLO is a CDC, and binds and forms pore-like structures on cholesterol crystals in a cell-free system [9, 11], so endometrial cell cholesterol content may influence the cellular sensitivity to PLO. Indeed, stromal cells contained more cholesterol than epithelial cells (Fig. 6A). As such, we considered if reducing stromal cell cholesterol might protect against the cytolytic activity of PLO. Concentrations of stromal cell cholesterol were reduced by 24-h treatment with 1000 μM methyl-β-cyclodextrin, but not following treatment with α-cyclodextrin or γ-cyclodextrin (Fig. 6B). Treatment of stromal cells with methyl-β-cyclodextrin afforded a concentration-dependent protection against 15 HU rPLO, with 1000 μM methyl-β-cyclodextrin abrogating cytolysis; stromal cytolysis was also reduced by 1000 μM γ-cyclodextrin, but not α-cyclodextrin (Fig. 6C).

Cellular Responses to *T. pyogenes*

The cellular responses to *T. pyogenes* and PLO were explored because endometrial pathology associated with isolation of *T. pyogenes* involves inflammation as well as endometrial tissue damage [5, 8]. Supernatants of in vitro organ cultures of endometrium accumulated IL-1β, IL-6, and IL-8 following 24-h treatment with 10^3–10^7 cfu live *T. pyogenes* strain MS249 (Fig. 7A) or *T. pyogenes* Δ*plo* (Fig. 7B), and the responses were of similar magnitude to the 1 μg/ml LPS positive control; the *T. pyogenes* cfu after treatment did not differ significantly from the input cfu following 24-h culture in culture medium. However, there was no detectable accumulation of IL-1β, IL-6, or IL-8 after 24 h treatment of in vitro organ cultures of endometrium with 0.003–30 HU rPLO (Fig. 7C).

Endometrial stromal and epithelial cell supernatants also accumulated more IL-6 after 24-h treatment with heat-killed *T. pyogenes* equivalent to 1000 MOI compared with control culture medium (Fig. 8A). However, stromal or epithelial cell supernatants did not accumulate detectable amounts of IL-6 or IL-8 after 24-h treatment with 0.003–30 HU rPLO (Fig. 8B). The concentrations of IL-1β were below the limits of detection of the assay. Similarly, the concentrations of CXCL1, CXCL2, and CXCL3 were below the limits of detection of the assays for stromal or epithelial cells treated with rPLO; although the cells responded to 1 μg/ml LPS (stroma, 761.3 ± 92.3, 2693.3 ± 386.5, and 1588.3 ± 184.3 pg/ml, respectively; epithelium, 2283.6 ± 164.2, 2254.0 ± 474.4, and 3334.0 ± 250.3 pg/ml, respectively; $P < 0.05$, ANOVA).

The absence of an inflammatory response to PLO, indicative of innate immunity, was not limited to this particular CDC, because concentrations of IL-1β, IL-6, or IL-8 were all below the ELISA limits of detection for stromal cells treated with 0.003–30 HU SLO.

To examine if hematopoietic cells might generate inflammatory responses to PLO, PBMCs were treated with 0.003–3 HU rPLO for 24 h. However, while there were the expected cellular responses to LPS with increased accumulation of IL-1β, IL-6, and IL-8, there was no detectable accumulation of IL-

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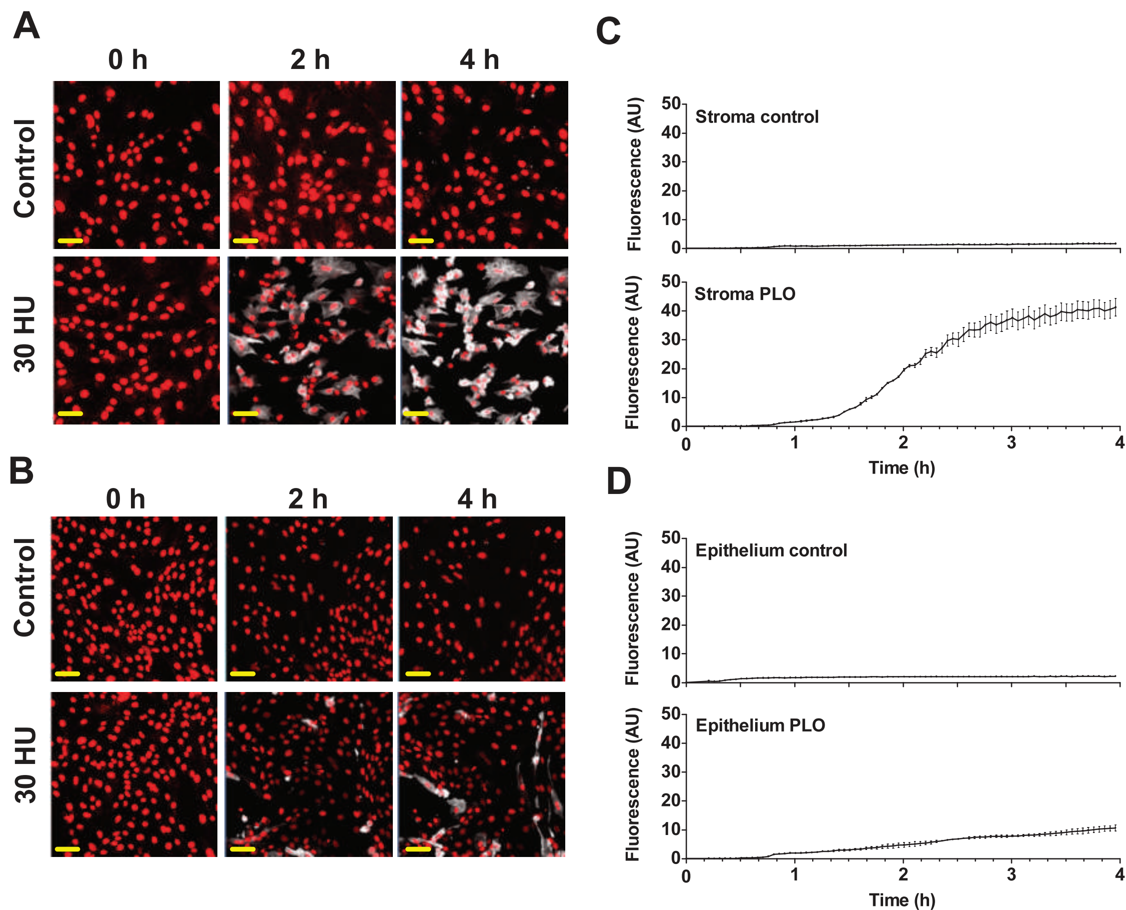

FIG. 5. Kinetics of endometrial cytolysis by PLO. Representative confocal microscope images of three independent experiments using stromal (**A**) and epithelial (**B**) cells treated with Hoechst 33342 (red nuclei) and fluorescently labeled phalloidin (white actin), in control culture medium or medium containing 30 HU PLO for the indicated times; bar = 50 μm. Stromal (**C**) and epithelial (**D**) cell florescence intensity measured by confocal microscopy every 3 min in arbitrary units. Data are presented as mean (SEM) of three independent experiments.

1β, IL-6, or IL-8 in the supernatant of cells treated with rPLO (Supplemental Fig. S5A), and only the highest concentration of rPLO reduced PBMC survival (64.0 ± 3.4 % of control, $P < 0.05$, ANOVA; Supplemental Fig. S5B).

DISCUSSION

The present study explored the mechanisms of how *T. pyogenes* causes tissue damage in the endometrium after parturition. The *plo* gene was conserved and the gene promoter was similar among multiple clinical isolates of *T. pyogenes*. The BFF from each bacterial isolate caused hemolysis and cytolysis of endometrial stromal and epithelial cells. The cytolytic agent in BFF was PLO, because a specific anti-PLO antibody abrogated the cytolytic activity of BFF, and a *plo*-deletion mutant of *T. pyogenes* had no cytolytic activity. Endometrial stromal cells were markedly more sensitive than epithelial cells to PLO-mediated cytolysis, and stromal cells were also more sensitive to PLO than hematopoietic immune cells or PBMCs. The stromal cell sensitivity to PLO may be due to these cells containing more cholesterol than epithelial cells, and reducing cellular cholesterol using cyclodextrins protected the stromal cells against PLO-mediated cytolysis. Surprisingly, the endometrial tissue or cells, hematopoietic immune cells, or PBMCs did not mount detectable inflammatory responses to PLO, although they responded to bacteria by secreting cytokines and chemokines. In summary, stromal cell sensitivity to PLO explains how *T. pyogenes* causes endometrial pathology once the protective epithelium is lost after parturition to exposure the underlying stroma.

T. pyogenes commonly causes severe uterine disease in postpartum dairy cattle, with tissue damage and inflammation of the endometrium [2, 5, 8, 9]. In one study, *T. pyogenes* was isolated from 42% of swabs collected from the endometrium of dairy cows 1–4 wk postpartum, and was significantly associated with uterine disease [36]. Similarly, in the present study, *T. pyogenes* acted as a primary pathogen and caused clinical endometritis with the discharge of pus when bacteria were infused into the uteri of dairy cows. However, most uterine infections involve multiple bacteria, both culturable and nonculturable [2, 37]. In one field study, only 30% of uterine lavage samples from animals with postpartum metritis yielded DNA attributable to the presence of *T. pyogenes*, and 21% of animals without signs of metritis possessed DNA from the bacterium [38]. In another study by the same group, *T. pyogenes* DNA was not identified from uterine flushings collected from postpartum cattle, with or without metritis [37]. Interactions between multiple species of bacteria are likely important in the pathogenesis of clinical disease of the uterus [2, 37]. However, the present study focused on the mechanisms linking *T. pyogenes* to disease in the endometrium, because isolation of the bacterium is correlated with the amount of pus in the female genital tract, and with the severity of inflammation in the stromal compartment of the endometrium [5, 36].

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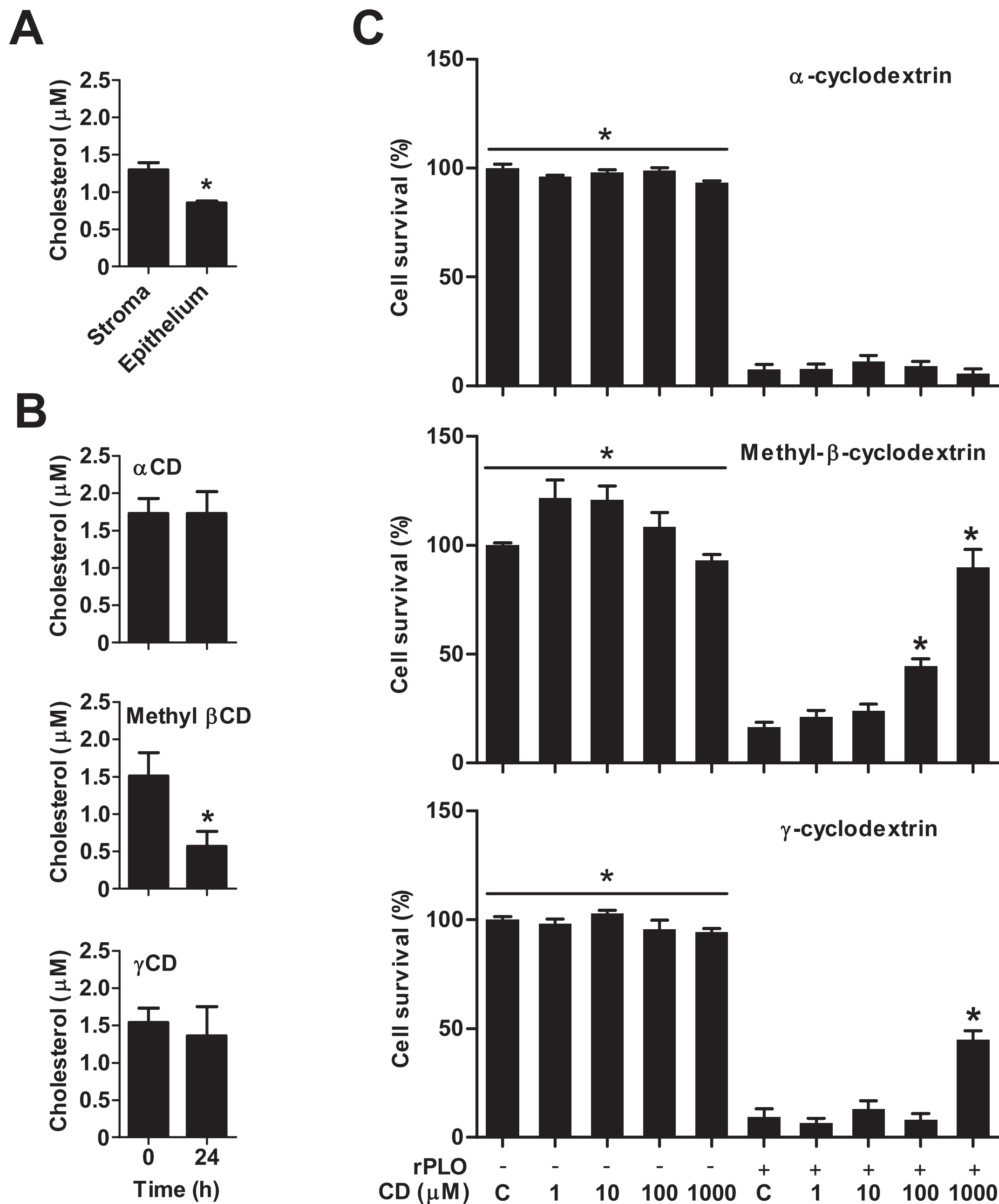

FIG. 6. Cellular cholesterol concentration and PLO-mediated cytolysis of endometrial cells. **A**) The cellular cholesterol concentration of 10^5 stromal and epithelial cells was determined by cholesterol assay. Data are presented as mean (SEM) of three independent experiments. Significant differences were determined by independent *t*-test; $*P < 0.05$. **B**) Stromal cells were treated for 0 and 24 h with α-, methyl-β-cyclodextrin or γ-cyclodextrin (CD) before measuring cellular cholesterol concentration. Data are presented as mean (SEM) of three independent experiments. Significant differences were determined by independent *t*-test; $*P < 0.05$. **C**) Stromal cells were treated with control culture medium (C) or medium containing the indicated concentrations of α-cyclodextrin, methyl-β-cyclodextrin, or γ-cyclodextrin for 24 h before treatment with control culture medium (–) or medium containing 15 HU rPLO (+) for 24 h, and cell survival was monitored by MTT assay. Data are expressed as a percent of control, and are presented as mean (SEM) of four independent experiments. Significant differences from cells in PLO were determined by ANOVA and Dunnett pairwise *t*-test; $*P < 0.05$.

The secreted pore-forming toxin PLO is considered the primary virulence factor of *T. pyogenes* [9], and the *plo* gene was expressed by all 57 isolates of *T. pyogenes* in a survey of postpartum uterine disease [19]. In the present study, the *plo* gene sequence was identical, and the *plo* gene promoter sequence was highly conserved among 12 clinical isolates collected from different farms and countries. The BFF from each of the isolates caused hemolysis, and caused cytolysis of endometrial and hematopoietic cells. There was some variation in the hemolytic activity of the BFF among the *T. pyogenes* isolates, which might relate to minor nucleotide variations in the *plo* gene promoter. However, this variation in hemolysis from bacteria grown in vitro may not reflect pathogenicity in vivo. Moreover, the endometrial cytolytic potency of BFF from these isolates did not differ significantly when the BFF were normalized to the same HUs of toxin. As well as finding PLO protein in BFF, PLO was essential for cytolysis, because a specific antibody against PLO blocked the hemolytic and endometrial cytolytic activity of BFF. Furthermore, deletion of *plo* prevented hemolysis and abrogated endometrial cytolysis

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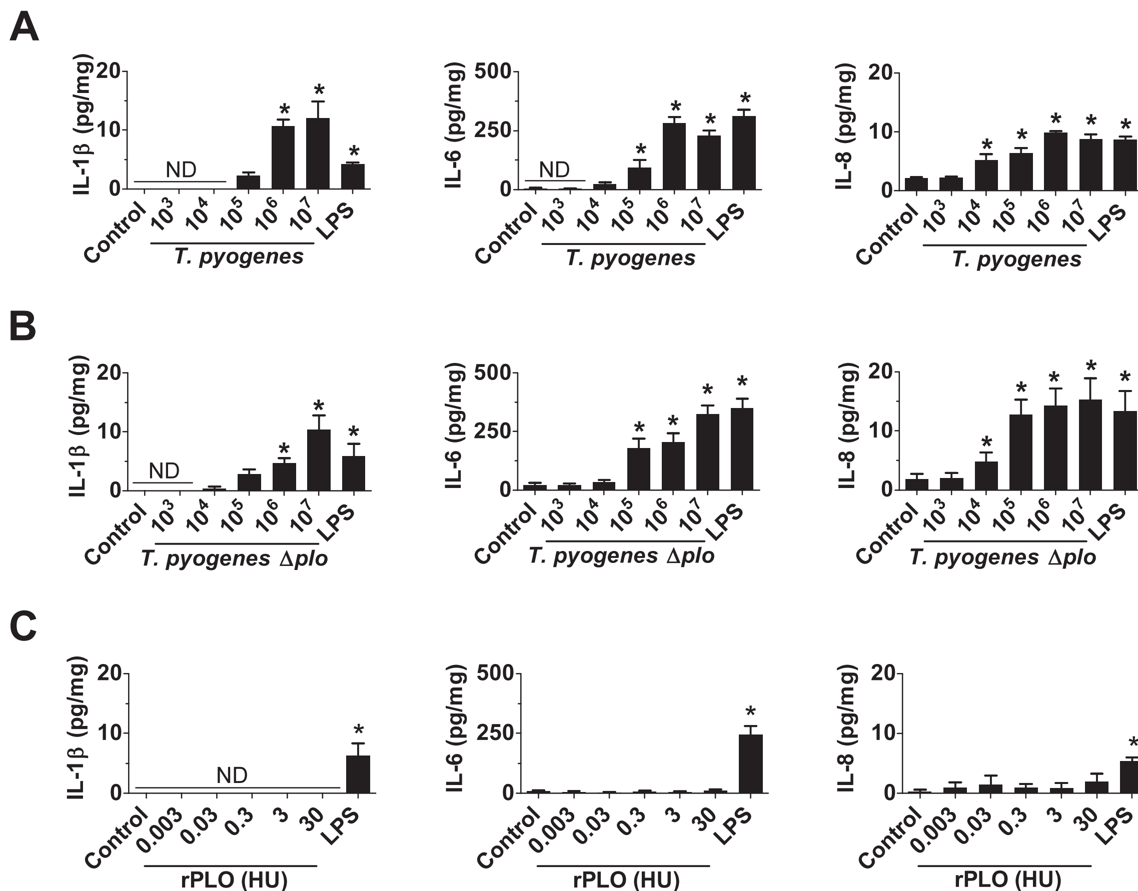

FIG. 7. Endometrial tissue responses to *T. pyogenes* and PLO. In vitro organ cultures of endometrium were treated for 24 h with control culture medium or medium containing 10^3–10^7 live *T. pyogenes* (**A**), 10^3–10^7 live *T. pyogenes* Δ*plo* (**B**), or 0.003–30 HU rPLO (**C**), with culture medium containing 1 μg/ml LPS used as a positive control in each case. Concentrations of IL-1β, IL-6, and IL-8 in supernatants were measured by ELISA. Data were normalized to the weight of each tissue in culture, and presented as mean (SEM) pg/mg tissue from four independent experiments. Significant differences from control were determined by ANOVA and Dunnett pairwise *t*-test; $^*P < 0.05$. ND, values below the limits of detection.

by live *T. pyogenes*. In a mouse peritonitis model, PLO was also important for *T. pyogenes* virulence [12], and, in other species of bacteria, mutation of genes in bacteria that encode CDCs also reduced their virulence [39, 40].

The most striking observation in the present study was the difference in sensitivity among host cells to PLO. The BFF from each of the 12 isolates of *T. pyogenes* consistently caused more stromal then epithelial cytolysis. Furthermore, endometrial stromal cells were markedly more sensitive to rPLO-mediated cytolysis than epithelial cells, monocytes, neutrophils, or lymphocytes. More detail about the kinetics of PLO action on endometrial cells was obtained using confocal microscopy to monitor fluorescence of phalloidin bound to F-actin following pore formation in plasma membranes [30]. More stromal than epithelial cells fluoresced, and the rate of permeation of stromal cells was more rapid than for epithelial cells. Therefore, we suggest that, once the endometrial epithelium is disrupted, PLO-mediated stromal cytolysis is a key step in the pathogenesis of postpartum endometritis.

The sensitivity of stromal cells and/or the resistance of epithelial cells to PLO-mediated cytolysis have biological relevance, because the columnar epithelium of the endometrium forms a physical barrier between the uterine lumen and the underlying stroma. However, after parturition, the epithelial layer is disrupted and invading microbes access the underlying stroma [1, 3–5]. The epithelium is disrupted by trauma during parturition and sloughing of the surface of the uterine caruncles about 12–14 days postpartum [3, 4]. These events are followed by cell proliferation and regeneration of the epithelium, which is completed between 20 and 30 days after parturition [3, 4]. Interestingly, the highest concentrations of *T. pyogenes* in the endometrium are detectable during the second and third week postpartum, prior to regeneration of the epithelium [41]. Once the stroma is colonized with bacteria, *T. pyogenes* infection and endometritis appear to persist [2, 5]; indeed, the tissue damage associated with infection may delay the re-epithelialization of the endometrium and foster a chronic infection. However, compartmentalization by an intact endometrial epithelium, mucus, and antimicrobial peptides likely prevents new infections of the endometrium by *T. pyogenes* beyond 20 days postpartum. Indeed, mechanical disruption of the endometrial epithelium of late-lactation dairy cows is important to be able to generate endometritis in the bovine *T. pyogenes* challenge model, as used in the present study [26]. The ability of *T. pyogenes* to cause pathology in the denuded endometrium is similar to the discovery of endometrial pathogenic *E. coli* that is adapted to the bovine endometrium [6]. On the other hand, the resistance of epithelial cells to PLO is also an example of how basal tolerance against a microbe can be used as an innate defense strategy in animals [13].

Most members of the family of CDCs bind cholesterol in host cell plasma membranes and then oligomerize to form a pore [9, 10]. CDCs, including PLO, can even bind and form pore-like structures on cholesterol crystals in a cell-free system

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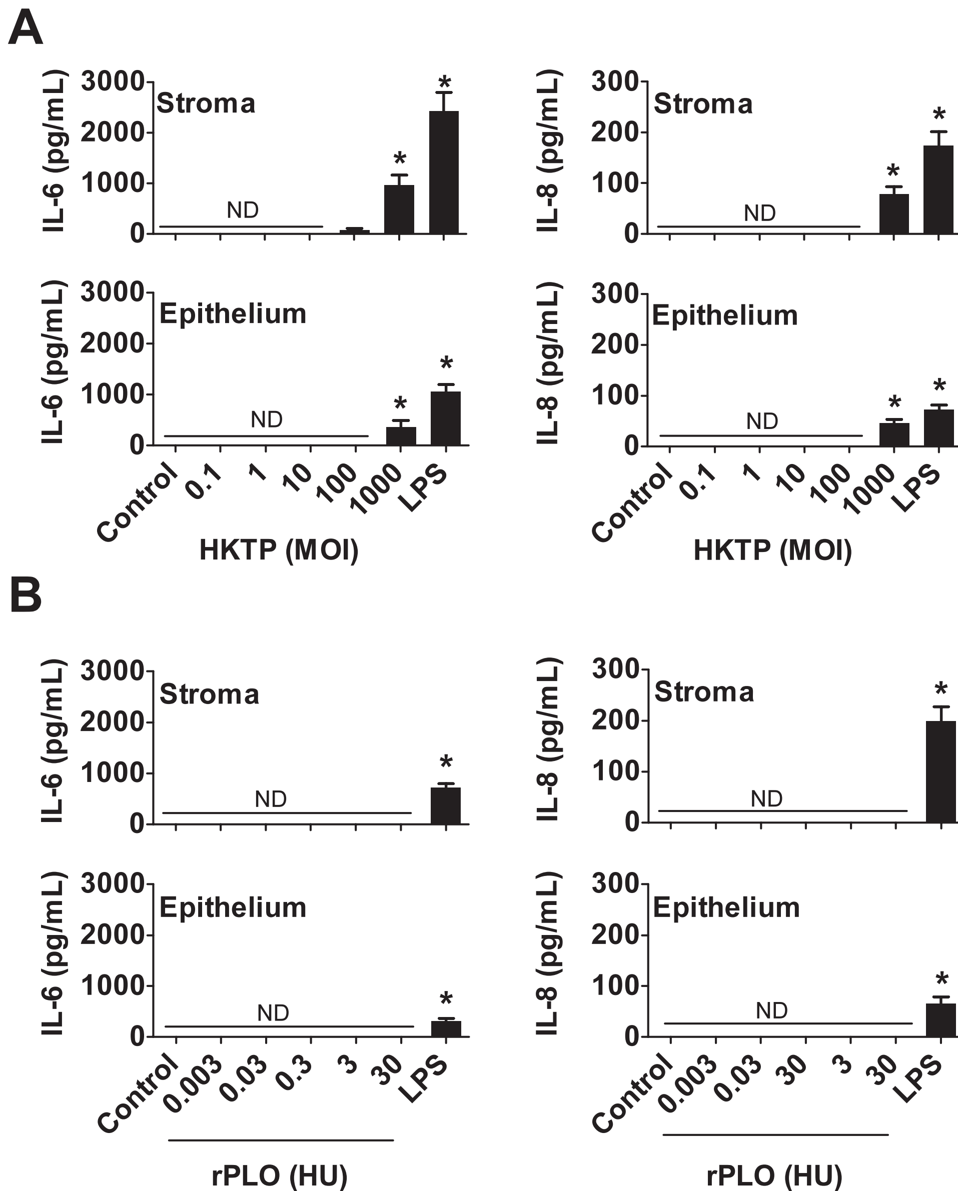

FIG. 8. Host cell inflammatory responses to *T. pyogenes* and PLO. Pure populations of primary bovine endometrial stromal and epithelial cells were treated for 24 h with 0.1–1000 MOI heat-killed *T. pyogenes* (HKTP; **A**) or 0.003–30 HU rPLO (**B**), with culture medium as a control, and 1 μg/ml LPS as a positive control. The concentrations of IL-6 and IL-8 that accumulated in the supernatant were measured by ELISA. Data are presented as mean (SEM) of four independent experiments. Significant differences were determined by ANOVA and Dunnett two-sided post hoc comparison with control; $^{*}P < 0.05$. ND, values below the limits of detection.

[11]. However, ring-shaped cyclodextrins, particularly methyl-β-cyclodextrin, bind and sequester cholesterol molecules [31]. Furthermore, because CDCs target cholesterol, the removal of cholesterol from cell membranes using cyclodextrins can reduce the hemolytic activity of CDCs [10]. In the present study, it was notable that stromal cells had higher cellular cholesterol content, and they were more sensitive to PLO-mediated cytolysis than epithelial cells. Furthermore, treating endometrial stromal cells with methyl-β-cyclodextrin also efficiently reduced cellular cholesterol content to a similar concentration found in epithelial cells. More importantly, addition of methyl-β-cyclodextrin protected the stromal cells against PLO-mediated lysis. As tissue damage is an important feature of uterine disease, reducing cytolysis using methyl-β-cyclodextrin might reduce the impact of *T. pyogenes*.

In addition to tissue damage, postpartum bacterial infection of the endometrium is also characterized by inflammation [7, 17]. Many highly conserved microbial molecules act as PAMPs that bind host cell pattern recognition receptors to stimulate inflammation [14]. Similarly, in the present study, *T. pyogenes* stimulated in vitro organ cultures of endometrium or endometrial cells to secrete inflammatory mediators indicative of an innate immune response. The response to *T. pyogenes* is readily explained by the presence of many common PAMPs in whole bacteria, such as lipopeptides and nucleic acids [14]. In addition, we also considered if PLO might be a PAMP, because other CDCs are thought to provoke an innate immune response [15, 16, 35]. However, in the present study, endometrial tissue, stromal or epithelial cells, or hematopoietic immune cells did not produce detectable amounts of cytokines or chemokines in response to PLO. The cells were capable of mounting

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inflammatory responses as the prototypical PAMP LPS simulated the release of cytokines and chemokines, as expected from previous observations of bovine endometrial cells [17]. Furthermore, the use of a prototypical CDC, SLO, did not provoke inflammatory responses by endometrial cells in the present study.

Taken together, the data from the present study indicate that *T. pyogenes* bacteria provoke a cellular inflammatory response, whereas PLO causes cytolysis. Conversely, the *plo*-deletion mutants of *T. pyogenes* do not appear to cause cytolysis, and rPLO did not stimulate the release of cytokines or chemokines. Of course, during clinical infections in the whole animal, *T. pyogenes* cause tissue damage and inflammation of the endometrium, often in association with other species of bacteria [2, 5, 8, 36]. These bacteria posses a range of PAMPs, including bacterial lipopeptides, nucleic acids, and LPS. The multiple PAMPs stimulate inflammatory responses by hematopoietic cells and by endometrial cells [14, 42]. In particular, endometrial epithelial and stromal cells mount inflammatory responses to LPS via Toll-like receptor (TLR) 4, and to bacterial lipopeptides via heterodimers of TLR2 with TLR1 or TLR6 [17, 43]. Although most PAMPs are highly conserved molecules from prokaryotes, it appears from our work that, while PLO is highly conserved, it is not a potent PAMP. In vivo, the presence of tissue damage caused by microbial virulence factors is also thought to increase the scale of the inflammatory response to bacterial infection beyond that attributable to the presence of PAMPs [44]. The release of damage-associated molecular patterns (DAMPs) from dying cells and damaged tissues are thought to stimulate inflammation when DAMPs are bound by pattern recognition receptors, including TLRs [45, 46]. However, the lack of inflammatory responses to cytolysis caused by PLO in the present study somewhat counters this concept of DAMPs stimulating inflammation. Although DAMPs were not specifically measured in the present study, PLO treatment caused cytolysis of all the types of cells examined, and yet no increase in the concentrations of inflammatory mediators was evident in cell or tissue culture supernatants. Further investigation of the DAMPs released when cells are exposed to CDCs, and examination of other types of cellular responses to CDCs, is warranted. Indeed, an insight into the latter was evident in the present study, because sublytic concentrations of PLO stimulated stromal cell proliferation. Similar results have been reported with *Staphylococcus* α-toxin, where transiently permeated keratinocytes were found to proliferate via an epidermal growth factor receptor-dependent pathway [47]. In the absence of inflammatory mediator responses to pore-forming toxins, cellular proliferation may be an example of an induced resistance mechanism to limit the impact of tissue damage in animals [13].

In conclusion, the marked sensitivity of stromal cells to PLO-mediated cytolysis provides an explanation for how *T. pyogenes* acts as an opportunistic pathogen to cause pathology of the endometrium once the protective epithelium is lost after parturition.

ACKNOWLEDGMENT

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Comparison of the *plo* gene. The DNA sequence coding for *plo* was obtained for 12 clinical isolates of *T. pyogenes* isolates and collected from the NCBI database (Genbank accessions: AAC45754, ADU04496, BAA86937), along with *Arcanobacterium hemolyticum* DSM 20595 thiol-activated cytolysin sequence (YP_003697395) and the *Streptococcus suis* thiol-activated cytolysin sequence YP_006358469). Sequences were used in phylogeny.fr webserver to present the data as a phylogenetic tree (Dereeper et al 2008 [27]).

Reference

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Supplemental Figure S2. Comparison of the *plo* promoter region. The *plo* gene promoter region from 12 clinical *Trueperella pyogenes* isolates was sequenced. The promoter region of isolates MS249, MS295, MS340 and MS348 were identical and used to comprise a consensus sequence, which was highly similar a sequence published previously (Rudnick et al, 2008 [48]). The colored bases correspond to the base in the sequence of isolates of that color (shown in the key) which varies from the consensus sequence. The regions described by Rudnick et al (2008 [48]) as direct repeats DR1, DR2 and DR3 are identical; in both the regions described as promoters P1 and P2 there was a transposition in nucleotides 1 and 2.

Reference

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Supplemental Figure S3. Characteristics of *Trueperella pyogenes*. (A) Bacterial growth curves were generated for each of 12 *T. pyogenes* clinical isolates as determined by measuring OD_{600} by spectrophotometer every 2 h for 24 h. Data are presented as mean (SEM) of 3 replicates for each isolate. (B) Hemolytic activity was measured for bacterial free filtrates (BFF) collected from each isolate at each indicated time point during growth. Data are presented as mean (SEM) of 3 BFF for each isolate. (C) Hemolytic activity in hemolytic units (HU) of bacteria-free filtrate (BFF) collected after 16 h growth of each of 12 *T. pyogenes* clinical isolates. Data are presented as mean (SEM) of 3 replicates for each isolate. (D) Pyolysin (PLO) protein was detected in BFF collected after 8, 16, 20 and 24 h bacterial growth. The BFF were dialyzed and concentrated, prior to SDS PAGE and Western blot using αPLO goat serum. Typical images are shown for isolate MS249.

Supplemental Figure S4. Endometrial cell survival after treatment with *T. pyogenes* Δ*plo*. Cell survival was calculated from MTT assays of (A) stromal and (B) epithelial cells after treatment with 1 to 100 multiplicities of infection (MOI) live *T. pyogenes* Δ*plo* for 24 h. Data presented as mean (SEM) of 4 independent experiments. Values did not differ from control following ANOVA.

Supplemental Figure S5. Peripheral blood mononuclear cell responses to pyolysin. (A) Peripheral blood mononuclear cells were isolated and treated with control culture medium, or medium containing 0.003 to 3 HU recombinant pyolysin (rPLO), or culture medium containing 1 µg/ml ultrapure lipopolysaccharide (LPS) as a positive control. The concentrations of IL-1β, IL-6, and IL-8 in supernatants were measured by ELISA. (B) Cell survival was evaluated by MTT assay and expressed as a percentage of cells in control culture medium. Data presented as mean (SEM) of 4 independent experiments. Values differ from control following ANOVA and Dunnett's's two sided comparison against control, * $P < 0.05$

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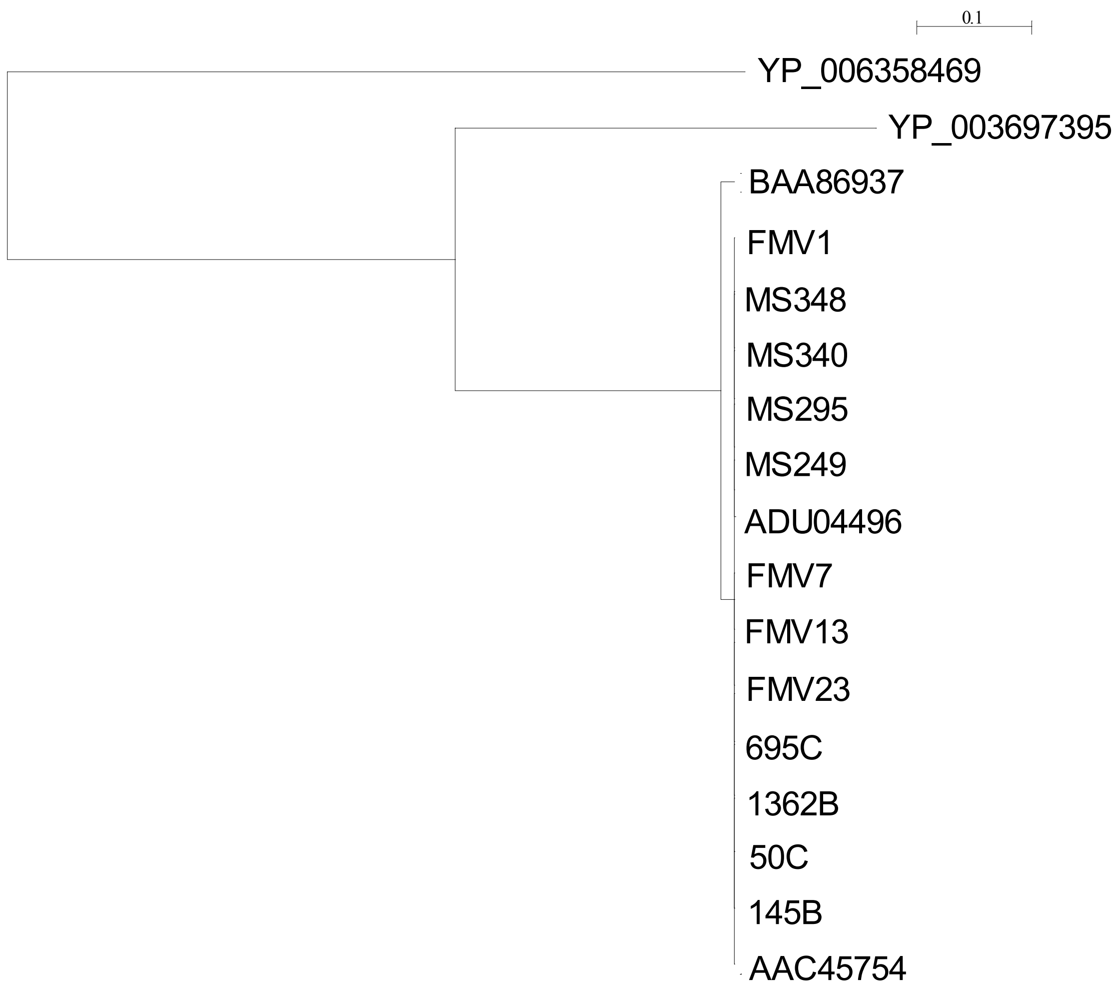
0.1
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FMV1
MS348
MS340
MS295
MS249
ADU04496
FMV7
FMV13
FMV23
695C
1362B
50C
145B
AAC45754

```
A A A t T GC T CG C t C T a
CGCTAGTGGTGACGGCGCCCCTCTGCGCAGCGTTGCTGCCATTACCAATTTATGGTACTAGCAGGTCA

T T G a C
TTTCAGAAATTGCAAGATATTGACAGGTTATCTCGAGTGTT TTCCCAGCAAAGGCGTGGTGAAAAAAT

C - T G A TG G
GCGATTTCAACGCTTATGCGATGCGTGAAAAAGCGCGTTTACCTGCGGGTTTATATGGCTATCGGGAG

C C G CG
ATTTAAGGGTGTGGGAAAAATGAGTCGCGCCAAT ATAGTATTGACTTTATcTTAATAGTGTGCATTCG

A A A A t t
CTTCATGgGCGAGCATTTTTGCTGGTCAATATCCCGTTTTCGGGCGGGCGATTTTTGCTGGCTAGTCA

c a t
GTGGTGAAGGTAACCTTTTTCTTGAGACCTG CGGTGTGAGGTCTGTAAATTCCAGGGGCTACGTGTGC

t
GCTCAATACGCTATAGTGAAAAAATATTTTTGTGGCTATAAGAGCTGTTAATAAGCTGATCTGACTCG

t A GA
TTGACATCGCCTTTTACACGTGTACTATCG AAGTATCTCCAGAGAGTTGGAGCTACCTTATTCGAGGT

A c
TGAGTTAA-GGAAGGCGGAAGGAA AATG...
```

Key:

SP-1,SP-2,SP-3,SP-4,FMV-1,FMV-7,FMV-13,FMV-21

SP-1,SP-3,SP-4,FMV-1,FMV-7,FMV-13,FMV-21

SP-1,SP-4,FMV-1,FMV-13

SP-1, SP-4, FMV-13

SP-1, SP-4

FMV-1, FMV-7, FMV21, SP3

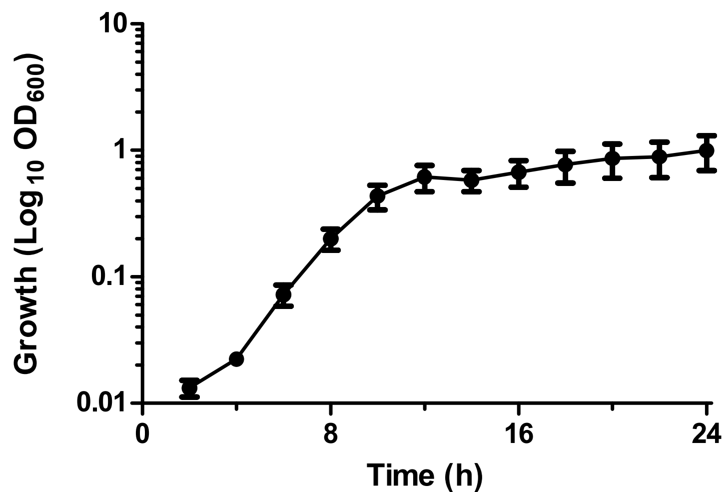
FMV-7, FMV-21

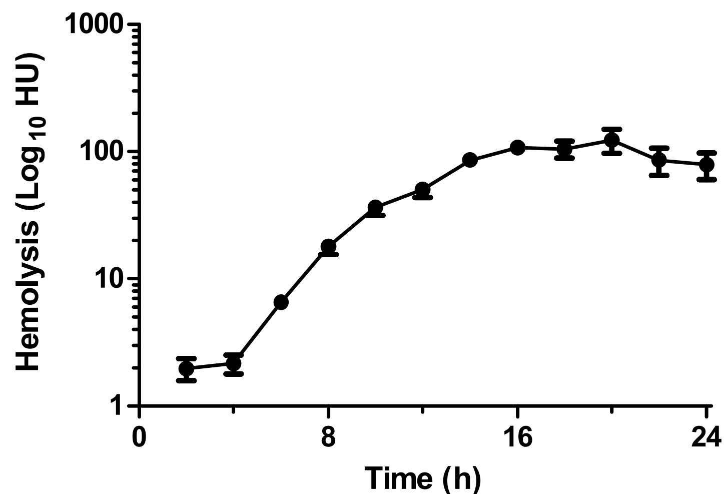
FMV-1

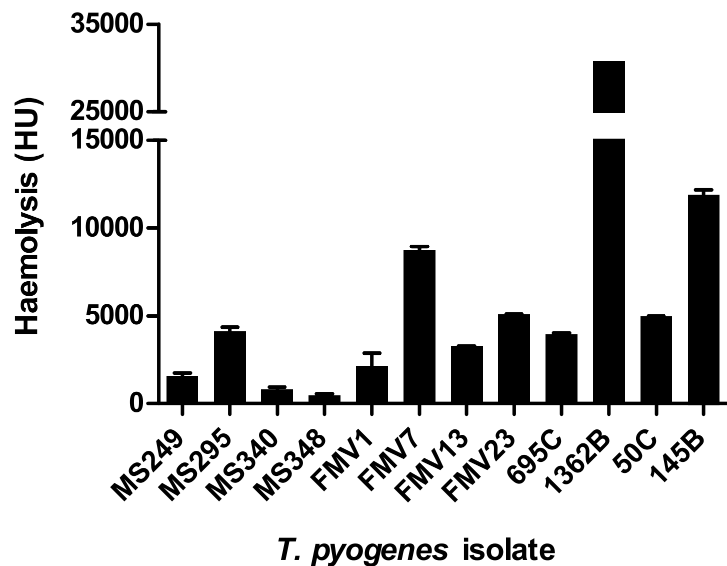
FMV-13

FMV-7

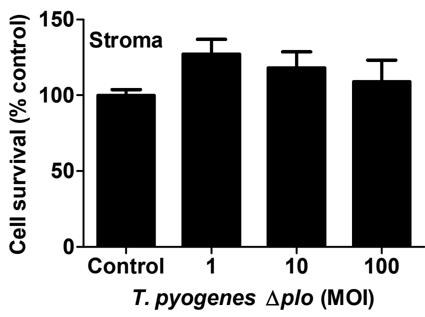
SP-3

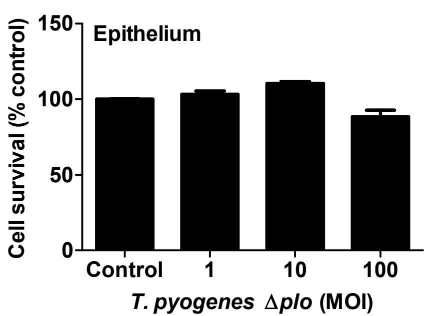

A
Growth (Log$_{10}$ OD$_{600}$)
10
1
0.1
0.01
0
8
16
24
Time (h)


B
Hemolysis (Log$_{10}$ HU)
1000
100
10
1
0
8
16
24
Time (h)

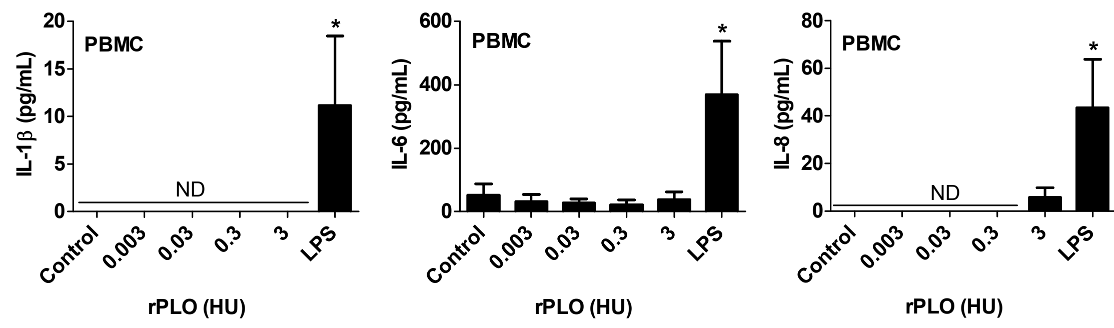

C
Haemolysis (HU)
35000
25000
15000
10000
5000
0
MS249
MS295
MS340
MS348
FMV1
FMV7
FMV13
FMV23
695C
1362B
50C
145B
T. pyogenes isolate

D
Time (h)
8
16
20
24
PLO 55 kDa

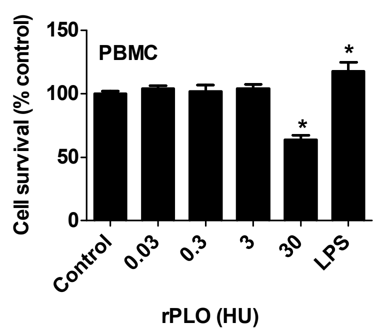

A
Stroma
Cell survival (% control)
150
100
50
0
Control
1
10
100
T. pyogenes Δplo (MOI)


B
Epithelium
Cell survival (% control)
150
100
50
0
Control
1
10
100
T. pyogenes Δplo (MOI)

A



B



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SUPPLEMENTAL TABLE S1

Supplemental Table S1. Primer sequences and restriction enzyme sites used in *plo* mutagenesis.

Oligonucleotide primer sequence (restriction site underlined)	Restriction site
KanRF: GTGAATTCTACACATCTCAACCAT	*Eco*RI
KanRR: CCAGAATTCCAACCAATTAACCAA	*Eco*RI
PloUF: GTGTCGACCCTCCTGTTCAG	*Sal*I
PloUR: GCCGAATTCAGGTCAACTTTATC	*Eco*RI
PloDF: AAGGAATTCCGTACACCCAAG	*Eco*RI
PloDR: TCGTCTAGATACGCAGAATCATGAG	*Xba*I

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