HHS Public Access

Author manuscript

Reproduction. Author manuscript; available in PMC 2021 December 14.

Published in final edited form as:

Reproduction. ; 163(1): 1–10. doi:10.1530/REP-21-0308.

Inflammatory Response Elicited by *Ureaplasma parvum* colonization in human cervical epithelial, stromal, and immune cells

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Abstract

Ureaplasma parvum is a commensal bacterium in the female reproductive tract but has been associated with pregnancy complications such as preterm prelabor rupture of membranes and preterm birth (PTB). However, the pathologic effects of *U. parvum* in the cervix, that prevents ascending infections during pregnancy, are still poorly understood. To determine the impact of *U. parvum* on the cervix, ectocervical (ecto) and endocervical (endo) epithelial and stromal cells were incubated with *U. parvum*. Macrophages were also tested as a proxy for cervical macrophages to determine the antigenicity of *U. parvum*. The effects of *U. parvum,* including influence on cell cycle and cell death, antimicrobial peptide production, epithelial-to-mesenchymal transition (EMT), and inflammatory cytokine levels, were assessed. *U. parvum* colonized cervical epithelial and stromal cells 4 hours post-infection. Like uninfected control, *U. parvum neither* inhibited cell cycle progression and nor caused cell death in cervical epithelial and stromal cells. *U. parvum* increased the production of the antimicrobial peptides (AMPs) cathelicidin and human β-defensin 3 and exhibited weak signs of EMT evidenced by decreased cytokeratin 18 and increased vimentin expression in cervical epithelial cells. *U. parvum* induced a pro-inflammatory environment (cytokines) and increased MMP-9 in cervical epithelial cells but promoted pro- and anti-inflammatory response in cervical stromal cells and macrophages. *U. parvum* may colonize the cervical epithelial layer, but induction of AMPs and anti-inflammatory response may protect the cervix and may prevent ascending infections that can cause preterm birth. These findings suggest that *U. parvum* is a weak inducer of inflammation in the cervix.

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Author contribution statement

OAGT and TK conducted the experiments. OT performed data analysis and drafted the manuscript. OAGT, RM, RBP, KLV conceived the project and designed experiments. RM provided funding. RM, TK, RBP, KLV, PMB helped with data analysis and interpretation and prepared the manuscript.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Keywords

antimicrobial peptide; cell death; cervical remodeling; epithelial-to-mesenchymal transition; infection; pregnancy

Introduction

The cervix protects the developing fetus from pathogenic microorganisms present in the vagina (Nott *et al.*, 2016; Vink and Feltovich, 2016). To maintain this function, the cervix undergoes extensive remodeling throughout pregnancy, starting as a closed, firm structure, which softens throughout pregnancy and shortens and dilates during labor and delivery. (Sennstrom, 2000; Vink and Myers, 2018). Infection and inflammation in the cervix may compromise its integrity leading to premature cervical remodeling and preterm birth (PTB) (Read *et al.*, 2007; Holt *et al.*, 2011; Vink and Feltovich, 2016; Vink and Mourad, 2017).

Ascending infections from the vagina to the uterine cavity account for almost 40% of preterm births (Goldenberg *et al.*, 2008). Intrauterine infections are usually polymicrobial in nature. However, the most isolated and detected microorganisms in the amniotic cavity are genital mycoplasmas particularly *Ureaplasma parvum* and *Ureaplasma urealyticum* (Sweeney *et al.*, 2017; Tantengco and Yanagihara, 2019; Sprong *et al.*, 2020). These bacteria have been associated with poor maternal outcomes such as preterm premature rupture of membranes (PPROM) and preterm birth (PTB) (Kacerovský *et al.*, 2009; Viscardi, 2010; Musilova *et al.*, 2016; Rittenschober-Böhm *et al.*, 2018). However, it is still unclear if *Ureaplasma* spp. are a cause of PPROM and PTB or if they simply are associated. This inconsistency on the role of *Ureaplasma* spp. in PPROM and PTB may be due to the methodological differences in the previous studies. Several clinical studies detect *Ureaplasma* spp., while others specifically detect *U. parvum* and *U. urealyticum*. The use of different species, serovars, dose, and site of inoculation in animal studies could have also confounded the association between *Ureaplasma* spp. infection and PTB. In vivo mice studies have shown that vaginal inoculation of *U. parvum* in an otherwise healthy cervix/vagina resulted in PTB rates (~10%) similar to that in women, however the PTB rates increased when the cervix was damaged or when *Ureaplasma* was directly inoculated inside the amniotic cavity (Motomura *et al.*, 2020; Pavlidis *et al.*, 2020). These reports suggest that damage to the cervical epithelial barrier can promote the ascent of *Ureaplasma* spp.

The lower rate of PTB in mice with an intact, healthy cervix, when compared to those with a damaged cervix, suggests that *Ureaplasma* spp. alone do not elicit a sufficient pro-inflammatory response to promote preterm labor-associated changes in cervical cells (Pavlidis *et al.*, 2020). Although previous animal and clinical studies have shown that *U. parvum* infection may promote an inflammatory response in the cervix (Horowitz *et al.*, 1995; Choi and Roh, 2014; Liu *et al.*, 2014; Motomura *et al.*, 2020; Pavlidis *et al.*, 2020), most of these studies focused primarily on pro-inflammatory cytokines and did not investigate the anti-inflammatory cytokine production of the cervix in response to *Ureaplasma* spp. infection. In a retrospective PPROM cohort study, Kacerovsky et al reported that *Ureaplasma* spp. colonization is associated with a significant increase in both

pro- and anti-inflammatory cytokines in the amniotic fluid suggestive of a balanced immune response in the intrauterine cavity. Moreover, *Ureaplasma* spp. colonization has not been associated with decreased gestational age at delivery (Kacerovsky *et al.*, 2013).

Previous studies have shown that different cellular mechanisms, such as inhibition of cell cycle progression and cell death, alteration of antimicrobial defense mechanisms, and epithelial-to-mesenchymal transition (EMT), can compromise cervical integrity (Allaire *et al.*, 2001; Hassan *et al.*, 2009; Gordon and Mowa, 2019). However, these cellular-level changes and types of innate immune responses have not been thoroughly investigated in the cervix in the presence of a bacterial infection. This study aimed to understand the pathologic effects of *U. parvum* in the resident cells of the cervix, particularly, epithelial, stromal, and immune cells. We also documented how colonization affects cell cycle, cell death, cellular transition, and antimicrobial response of cervical cells.

Materials and methods

Human ectocervical (ecto) and endocervical (endo) epithelial cell and cervical stromal cell (Stroma) cultures

Previously characterized immortalized ecto and endo cells (HPV E6/E7 cell immortalization system) and cervical stroma cells (hTERT cell immortalization system) from hysterectomy material from women with benign gynecological conditions were used in this study (Herbst-Kralovetz *et al.*, 2008; Tantengco *et al.*, 2021a–c). Ecto and endo cells were cultured in keratinocyte serum-free medium (KSFM), a culture medium highly selective for epithelial cells, supplemented with bovine pituitary extract (30 μg/mL), epidermal growth factor (0.1 ng/mL), $CaCl_2$ (0.4 mM) and primocin (0.5 mg/mL) (ant-pm-1; Invitrogen; San Diego, CA). The stromal cells were cultured with Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 medium (DMEM/F12; Mediatech Inc.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 10% penicillin/streptomycin (Mediatech Inc.), and 10% amphotericin B (Sigma-Aldrich, Inc.), at 37°C and 5% CO_2, and grown to 80% confluence. Cell culture protocols have previously been reported in our prior publications (Tantengco *et al.*, 2021b).

Human THP-1 macrophage culture

THP-1 monocytes were obtained from the American Type Culture Collection (ATCC TIB-202) and were cultured in RPMI 1640 Medium (ATCC modification) (A1049101, ThermoFisher Scientific) and supplemented with 0.05 mM 2-mercaptoethanol (21985023, Gibco™, Carlsbad, CA) and 10% FBS (Sigma-Aldrich). The cells were grown until they reach a density of 1×10^6 cells/ml. The cells were then centrifuged at 1000 rpm for 5 minutes. THP-1 monocytes were seeded in a 48-well plate (50,000 cells per well) and were differentiated to THP-1 macrophages by incubating in culture medium containing 100 mM phorbol 12-myristate 13-acetate for three days at 37°C and 5% CO_2 and grown to 80% confluence as described previously (Bowdish *et al.*, 2005).

Bacterial strain and culture conditions

U. parvum (ATCC® 700970™) were obtained from the American Tissue Culture Collection (ATCC). *U. parvum* was propagated in UMCHs medium (Namba *et al.*, 2010): *Mycoplasma* broth base (Becton, Dickinson and Co., Baltimore, MD) 1.47% (wt/vol), yeast extract (Becton, Dickinson and Co.) 2.5% (wt/vol), horse serum (Biowhittaker, Walkersville, MD) 20% (vol/vol), urea 0.04% (wt/vol), phenol red 0.001% (wt/vol), l-cysteine hydrochloride 0.01% (wt/vol), and penicillin G 1000 U/mL. *U. parvum* cultures were incubated for 16 – 18 h to obtain titers of $1 \times 10^9 – 1 \times 10^{11}$ color-changing units (CCU)/mL of viable bacteria. The corresponding amounts of *Ureaplasma* DNA were verified using genesig Std Real-time PCR detection kit, *Ureaplasma parvum* (Z-Path-U.parvum-std, American Research Products Inc., Waltham, MA) and amounted to $3 \times 10^7 – 3 \times 10^8$ copy numbers/mL.

Labeling of *U. parvum* Cells

U. parvum culture harvested after 16 – 18 h incubation in UMCHs medium were concentrated using Amicon® Ultra-15 Centrifugal Filter Units (UFC910024, 100 kDa; Merk Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) and was centrifuged at 4000 × *g* for 15 min. The concentrated *U. parvum* cells were labeled with DiOC18(3) (3,3'-dioctadecyloxacarbocyanine perchlorate – DiO; D275; Invitrogen, Carlsbad, CA, USA) by resuspending the concentrated *U. parvum* suspension with 100 μL of 100 μM DiO and were incubated at 37 °C for 30 min. To remove excess DiO, the *U. parvum* suspension was transferred to Amicon® Ultra-15 Centrifugal Filter Units and was centrifuged at 4000 × *g* for 15 min.

U. parvum Infection Assays

For immunocytochemical staining of the EMT markers cytokeratin 18 (CK-18, epithelial marker) and vimentin (mesenchymal marker) as well as antimicrobial peptides (AMP), ecto and endo cells were seeded at approximately 80% confluence in an eight-well glass slide and incubated at 37°C with 5% CO_2 for 24 h. Approximately 250 μL broths containing $10^9 – 10^{11}$ CCU/mL *U. parvum* were inoculated per milliliter of cell culture medium. The cells were stimulated for 48 h before staining for EMT markers and AMP.

To check for inflammatory cytokine production (ELISA), cell cycle and cell death (flow cytometry) EMT and AMP production (western blot [WB] analyses), ecto, endo, and stroma cells were seeded at approximately 80% confluence in a 6-well plate and incubated at 37°C with 5% CO_2 for 24 h. Approximately 250 μL broths containing $10^9 – 10^{11}$ CCU/mL *U. parvum* were inoculated per milliliter of cell culture medium. The cells were stimulated for 48 h before performing ELISA, flow cytometry, and WB.

Flow cytometry assays

Cell cycle analysis—Cell cycle analysis was performed by measuring DNA content to distinguish between different phases of the cell cycles. Fluorescence intensity, which directly correlated with the amount of DNA contained in a cell, was measured. Concurrent parameter measurements made it possible to discriminate between S, G2, and mitotic cells. As the DNA content doubles during the S phase, the intensity of the fluorescence increases, making

it possible to ascertain the effect of *U. parvum* infection on cell proliferation. Cell cycle analysis was performed using the flow cytometer, as described previously using the Coulter DNA Prep Reagents Kit (Beckman Coulter, Indianapolis, IN) (Tantengco *et al.*, 2021b). Briefly, cells were harvested by trypsinization and centrifuged for 5 min at 3000 × g. Cell pellets were washed with cold 1× PBS and centrifuged at 3000 × g for 5 min. Cell pellets were fixed with 500 μL 70% ethanol for 15 min. Cell pellets were washed with cold 1× PBS and centrifuged at 3000 × g for 5 min. Then, 500 μL of the prep stain was added to the tubes, vortexed, and run immediately on the CytoFlex flow cytometer (Beckman Coulter). After selecting for single cells, gating was set for the control cells and applied to histograms for each of the treatments in different cervical cells using CytExpert (Beckman Coulter).

Apoptosis and necrosis—To determine the population of cells undergoing apoptosis and/or necrosis, cells were stained using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & PI (Life Technologies, Carlsbad, CA) as reported previously (Tantengco *et al.*, 2021b). Briefly, cells were harvested after 48 h of *U. parvum* infection by trypsinization and centrifuged for 5 min at 3000 × g. Cell pellets were washed with cold 1× PBS and centrifuged at 3000 × g for 5 min. Pellets were resuspended in 100 μL 1× annexin binding buffer supplemented with 5 μL Alexa Fluor 488 Annexin V and 1 μL 100 μg/mL propidium iodide (PI). After a 15 min incubation, 400 μL annexin binding buffer was added, and samples were run immediately on the CytoFlex flow cytometer (Beckman Coulter). Unstained cervical cells were used as negative controls for gating. Data were analyzed using CytExpert software (Beckman Coulter).

Immunocytochemical staining of EMT markers (intermediate filaments CK-18 and vimentin) and antimicrobial peptides (cathelicidin, elafin, and human β-defensin 3)

Human, animal, and *in vitro* cell culture studies showed evidence of transition of cervical epithelial cells into a more mesenchymal phenotype. This transition is characterized by decreased expression of CK-18 and E-cadherin and increased expression of vimentin and N-cadherin and pro-inflammatory changes (Hassan *et al.*, 2009; Gordon and Mowa, 2019; Tantengco *et al.*, 2021c, d). Immunocytochemical staining for CK-18 (ab668; Abcam), vimentin (ab92547; Abcam, Cambridge, MA), cathelicidin (NBP1–76864, Novus Biologicals Inc., Littleton, CO), beta-Defensin 3 (NB200–117, Novus Biologicals Inc.), or elafin (MAB1747, R&D Systems, Inc., Minneapolis, MN) were performed 48 h post-infection with *U. parvum*. The dilution factor for primary antibodies was 1:500 for CK-18, 1:300 for vimentin, and 1:200 for cathelicidin, elafin, and human β-defensin 3. After each time point, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X, and blocked with 3% bovine serum albumin in PBS before incubation with primary antibodies overnight at 4°C. This protocol is adequate to remove non-specific binding of primary antibodies in our system, based on our previous studies (Tantengco *et al.*, 2021c, a). After washing with PBS, slides were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and diluted 1:400 in PBS for 1 h in the dark. Slides were washed with PBS, treated with NucBlue Fixed Cell Stains ReadyProbes Reagent (R37606; Thermo Fisher Scientific, Watham, MA), and then mounted using Mowiol 4 to 88 mounting medium (475904–100GM-M; Sigma-Aldrich, Inc.).

Western blot

Ecto and endo cells were infected with *U. parvum* for 48 h before lysis in RIPA lysis buffer with freshly added protease and phosphatase inhibitors (0.01%). The cell lysate was collected after scraping the culture plate, and the insoluble material was removed by centrifugation at 10,000 g for 20 minutes at 4°C. The concentration of protein in each cell lysate was determined using the BCA protein assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA). Equal amounts of protein (8 μg) from each sample were loaded onto a 10% SDS-PAGE gel and electrophoresed at 120 V. The resolved proteins were transferred to a polyvinylidene difluoride membrane using the iBlot transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% skim milk for 2 h at room temperature. Blots were incubated separately with antibodies against, β-actin (Sigma-Aldrich, A5441), CK-18 (Abcam, ab668), cathelicidin (NBP1–76864, Novus Biologicals Inc.), vimentin (Abcam, ab92547) at 4°C and shaken overnight. Blots were washed three times with TBS-T and incubated with an appropriate peroxidase-conjugated IgG secondary antibody for 1 h at room temperature. All blots were developed using chemiluminescence reagents (ECL Western Blotting Detection System, Amersham Piscataway, NJ, USA) in accordance with the manufacturer's recommendations, followed by autoradiography. Densitometry was performed to normalize the data for statistical analysis.

Enzyme-linked immunosorbent assay for GM-CSF, IL-6, IL-8, IL-10, and TNFα

Culture media collected from ecto, endo, stroma, and THP-1 macrophages 48 h post-infection were tested for human GM-CSF, IL-6, IL-8, IL-10, and TNFα using the BD OptEIA™ Human GM-CSF ELISA Set (555126, BD Biosciences, San Jose, CA), BD OptEIA™ Human IL-6 ELISA Set (555220, BD Biosciences), BD OptEIA™ Human IL-8 ELISA Set (555244, BD Biosciences), BD OptEIA™ Human IL-10 ELISA Set (555157, BD Biosciences), and Human TNF ELISA Set (555212, BD Biosciences), respectively. Standard curves were developed using duplicate samples of known-quantity recombinant proteins that were provided by the manufacturer. Sample concentrations were determined by relating the absorbance of the samples to the standard curve using linear regression analysis.

Statistical Analysis

Data were analyzed for significant differences using GraphPad Prism software version 7 (GraphPad Software, San Diego, CA). The Shapiro-Wilk test for normality was performed to check for the normality of the data. Parametric tests including one-way analysis of variance followed by the Tukey multiple comparison posthoc test and the t-test were used for comparison of the results for normally distributed data. Non-parametric tests, namely the Kruskal-Wallis test with Dunn's multiple comparison test and the Mann-Whitney U test were used for comparison of the results for data that were not normally distributed. Statistically significant differences are indicated by $p < 0.05$.

Results

U. parvum can be internalized by cervical epithelial and stromal cells

To determine if *U. parvum* can infect cervical epithelial and stromal cells, intracellular localization of DiO-labeled *U. parvum* was evaluated using immunocytochemical staining. CK-18 was used as marker for ecto and endo cells while vimentin was used for stroma. At 4 h post-infection, DiO-labeled *U. parvum* was observed in the cytosolic and perinuclear region with a clustered pattern in ecto, endo, and stroma cells (Fig. 1). These results showed that *U. parvum* can infect cervical epithelial and stromal cells in vitro.

U. parvum elicits antimicrobial response in cervical epithelial cells

Cervical epithelial cells were previously shown to produce AMP, including cathelicidin, elafin, and human β-defensin 3, in response to bacterial infection (Frew and Stock; Yarbrough *et al.*, 2015). It was also reported that AMP production is increased in patients with cervical shortening and preterm birth (Tribe, 2015; Hezelgrave *et al.*, 2020), therefore the AMP production of ecto and endo cells was quantified after 48 h infection with *U. parvum*. Immunocytochemical staining (ICC) and WB analysis showed a significant increase in cathelicidin staining in ecto cells infected with *U. parvum* ($p < 0.05$) (Fig. 2A, B, C). An increasing trend for cathelicidin was also observed in endo cells; however, it did not reach statistical significance (Fig. 2D, E, F). ICC staining also showed an increased human β-defensin 3 expression in ecto and endo cells. Elafin expression was increased in ecto but was decreased in endo cells (Fig. S1). These results demonstrate that cervical epithelial cells can increase AMP production in response to *U. parvum* infection.

U. parvum does not promote cell cycle arrest and cell death in cervical epithelial and stromal cells

Cell cycle arrest and cell death are important mechanisms that can prevent persistent bacterial infection, and this can be considered as an innate resistance by cells to minimize microbial spread. However, bacteria have developed mechanisms that can manipulate host cell death to allow bacterial colonization (Ashida *et al.*, 2014; Sixt, 2021), therefore, the effects of *U. parvum* colonization and cell cycle progression and cell death in cervical epithelial and stroma cells were analyzed using flow cytometry. *U. parvum* colonization promoted G1-S transition as shown by the significant increase in the population of ecto, endo, and stroma cells in S phase (Fig. 3A–C). *U. parvum* colonization did not induce apoptosis or necrosis in ecto and stroma but significantly decreased live endo cells ($p < 0.0001$) and significantly increased necrotic endo cells ($p < 0.0001$). However, necrotic cells only accounted for ~2% of the total endo cell population, which is negligible (Fig. 3 D–F). Collectively, these data showed that *U. parvum* did promote cell cycle arrest and did not induce cell death in cervical epithelial and stromal cells.

U. parvum induces minimal EMT in cervical epithelial cells

We previously reported that infectious and inflammatory stimuli may promote EMT in cervical epithelial cells (Tantengco *et al.*, 2021a, d, c), which may potentially compromise the cervical epithelial barrier functions and promote premature cervical remodeling due to

inflammation and increased presence of pro-inflammatory mesenchymal cells. EMT in ecto and endo cells was evaluated after *U. parvum* colonization using ICC and WB for CK-18 and vimentin. ICC analysis showed that *U. parvum* infection induced higher expression of vimentin and lower CK-18 expression in ecto (Fig 4A). WB analyses showed CK-18 and vimentin expression remain unchanged in ecto after *U. parvum* infection ($p < 0.05$) (Fig 4B–D). On the other hand, endo cells infected with *U. parvum* appeared to have lower expression of CK-18 and higher expression of vimentin compared to uninfected control (Fig. 5A, B). But densitometric analysis of WB did not show a statistically significant difference (Fig. 5C, D). These results suggested that *U. parvum* infection is a weak inducer of EMT and transitions are cell-type dependent. Ecto cells are more susceptible to EMT compared to endo cells at the dose of *U. parvum* used for stimulation in this study.

U. parvum promotes different inflammatory responses in cervical epithelial and stromal cells, and macrophages

Previous studies have shown that *U. parvum* infection can elicit inflammatory responses in maternal and fetal cells (Aaltonen *et al.*, 2007; Noda-Nicolau *et al.*, 2016; Potts *et al.*, 2016; Glaser *et al.*, 2017; Polettini *et al.*, 2018; Pavlidis *et al.*, 2020). The inflammatory response of cervical epithelial and stromal cells and macrophages was determined after infection with *U. parvum* for 48 h. In ecto cells, the increasing trend with MMP-9 after *U. parvum* infection did not reach statistical significance (Fig 6A, B). However, MMP-9 production by endo cells was significantly increased by *U. parvum* infection (Fig. 6 C, D). Pro-inflammatory markers were increased in ecto and endo cells after UP infection, with significantly increased IL-6 ($p < 0.05$) and IL-8 ($p < 0.05$) levels in ecto cells and GM CSF, IL-6, and IL-8 levels ($p < 0.0001$) in endo cells (Fig. 7)

In stroma cells, there was a mixed response with an increase in the proinflammatory cytokines IL-6 ($p < 0.01$) and IL-8 ($p < 0.05$), but also an increase in the anti-inflammatory IL-10 levels (Fig. 7). Similarly, in macrophages, exposure to *U. parvum* resulted in a significant increase in the pro-inflammatory cytokines IL-8 ($p < 0.05$) and TNFα ($p < 0.01$) while the anti-inflammatory cytokine IL-10 ($p < 0.05$) was also increased (Fig. 8). These results showed a cell-specific inflammatory response of cervical and immune cells after *U. parvum* infection.

Discussion

Cervical remodeling is a vital process that maintains pregnancy until term delivery (Yellon, 2017, 2020). Cervical infections are hypothesized to promote pathologic processes that may compromise the cervix, hasten the cervical ripening process, and predispose the tissue to preterm birth (Word *et al.*, 2007). We investigated these pathological processes in cervical cells in response to *U. parvum* infection. Our main findings include the following: 1) *U. parvum* can colonize cervical epithelial and stromal cells; 2) *U. parvum* colonization increased cathelicidin production in ecto and endo cells 3) *U. parvum* did not promote cell cycle arrest nor cause cell death in cervical cells; 4) EMT was not pronounced although there was a mild form EMT in ecto cells; 5) *U. parvum* induced a pro-inflammatory environment including an increase in matrix degrading MMP-9 levels in cervical epithelial

cells but promoted a mixed pro- and anti-inflammatory response in cervical stromal cells and human macrophages. In summary, *U. parvum* can colonize cervical cells and induce an inflammatory response; however, its antigenicity may not be sufficient to independently cause preterm birth.

Our study showed that *U. parvum* colonization does not result in cell death and may promote cervical cells to proliferate as signified by increased G1-S phase transition. Previous studies on *Ureaplasma* spp. showed similar results; Wolfs et al showed there was no induction of apoptosis in enterocytes while Silwedel et al reported inhibition of apoptosis in pulmonary endothelial cells (Wolfs *et al.*, 2013; Silwedel *et al.*, 2019). Several intracellular bacteria inhibit host cell apoptosis so they can persistently colonize the cells, which can facilitate their dissemination (Behar and Briken, 2019; FitzGerald *et al.*, 2020). The lack of apoptosis and necrosis in cervical cells is expected since *Ureaplasma* spp. are known to be part of the commensal microbiota in the cervicovaginal space (Kallapur *et al.*, 2013). However, this may also be a way for *U. parvum* to persistently colonize these cells for further dissemination in other gestational tissues.

U. parvum colonization increased the production of cathelicidin and human β-defensin 3 in cervical epithelial cells. Xiao et al. demonstrated that cathelicidin and human β -defensin 3 has antimicrobial activity against *Ureaplasma spp.* (Xiao *et al.*, 2014). Frew et al. showed that cathelicidin concentration is increased in cervicovaginal secretions of women with bacterial vaginosis. Increased cathelicidin production is expected to protect the cervix by killing the bacteria or by modulating the innate immune response (Frew *et al.*, 2014). Suff et al. showed that cervical gene delivery of human β-defensin 3 may boost innate immunity in the cervix and prevent *Escherichia coli* ascending infection-associated preterm birth (Suff *et al.*, 2020). Overall, the increase in AMP production in cervical epithelial cells may protect the cervix from *U. parvum* infection.

U. parvum infection may compromise the integrity of the cervical epithelial barrier by inducing EMT and inflammation in ecto and endo cells. EMT in the cervix has been shown to be involved in cervical remodeling during pregnancy. Parturition is associated with an increase in the expression of mesenchymal markers such as vimentin and N-cadherin, and a decrease in epithelial markers such as cytokeratin and E-cadherin (Hassan *et al.*, 2009; Gordon and Mowa, 2019). Our previous in vitro studies have shown that infection and inflammation cause EMT in cervical epithelial cells (Tantengco *et al.*, 2021d, c). However, the evidence of induction of EMT by *U. parvum* is not as strong as we have seen in our prior reports with LPS and TNFα. It is likely that *U. parvum* may promote EMT in ecto and endo cells but the constant exposure of cervical cells to high levels of progesterone during pregnancy may not allow full transition of these cells into proinflammatory mesenchymal cells to prevent cellular damage and inflammation (Tantengco *et al.*, 2021c). Our study also showed that *U. parvum* increased the levels of MMP-9 and pro-inflammatory cytokines, particularly IL-8, IL-6, and GM-CSF, in ecto and endo cells. This increase in collagenolytic enzymes and pro-inflammatory cytokines are hallmarks of cervical ripening (Sennstrom, 2000; Kelly, 2002; Choi *et al.*, 2009). These responses are likely not an effect of EMT but innate immune responses by cervical epithelial cells.

The cervical stromal cells and macrophages elicited both pro- and anti-inflammatory responses to *U. parvum* colonization. While these cells increased the production of pro-inflammatory cytokines, they also increased the IL-10 production in response to *U. parvum* colonization. IL-10 counteracts pro-inflammatory responses and modulates intracellular bacterial infections (Cyktor and Turner, 2011). It inhibits excessive Th1 and CD8+ T cell responses that contribute to tissue damage (Couper *et al.*, 2008). IL-10 can also reduce the production of pro-inflammatory cytokines (including IL-1α and β, IL-6, IL-12, IL-18, and TNF-α) (Moore *et al.*, 2001). Altogether, our data suggest that the increased IL-10 levels in cervical stromal cells and macrophages may counteract the inflammatory milieu and prevent further inflammation in the cervix.

This study had several strengths and limitations. This study showed the differential effects of *U. parvum* infection in cell cycle, cell death, antimicrobial and inflammatory (both pro- and anti-inflammatory) responses in ecto- and endocervical epithelial and stromal cells, and macrophages. Limitations of this study include the use of a single dose of *U. parvum* for all experiments and the use of one time point in assessing the cellular effects of *U. parvum* in cervical cells. However, the dose used in this study was within the pathologic dose of *Ureaplasma* spp. recovered from the cervical fluid of pregnancies complicated by preterm labor <37 weeks (Kacerovsky *et al.*, 2014; Musilova *et al.*, 2016). Additionally, this in vitro monoculture set-up does not fully mimic the in vivo tissue microenvironment where all cells are in physical contact and can communicate with each other. The effects observed in this experiment may change when these cells are allowed to communicate in culture together such as in microfluidic organ-on-a-chip or cervix tissue explants (Richardson *et al.*, 2020).

Overall, our results may explain the relatively low PTB rate in when *Ureaplasma* spp. is inoculated intravaginally in mice with a healthy cervix (Motomura *et al.*, 2020; Pavlidis *et al.*, 2020). An intact cervix has mechanisms to protect itself from premature cervical ripening by preventing inflammation and cell death induced by infection. We propose that *U. parvum* is not a strong inducer of inflammation in the cervix. It may compromise the integrity of the cervical epithelial barrier, but the cervix can mount innate immune responses such as antimicrobial peptide and anti-inflammatory cytokine responses that may counter the pathologic effects of *U. parvum* in the cervix. In summary, *U. parvum* infection alone is not enough to compromise an intact cervix and cause significant increase in preterm birth. We hypothesize that it may render the cervical cells more susceptible to infection by more antigenic pathogens such as *Escherichia coli* and *Gardnerella vaginalis*. This polymicrobial infection can fully compromise the cervix and ultimately result in microbial invasion of the amniotic cavity. When *U. parvum* invades the amniotic cavity, it can promote preterm birth and neonatal morbidity including bronchopulmonary dysplasia, intraventricular hemorrhage, necrotizing enterocolitis, and sepsis (Katz *et al.*, 2005; Viscardi and Kallapur, 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

Ourlad Alzeus G. Tantengco is an MD-PhD trainee in the MD-PhD in Molecular Medicine Program, supported by the Philippine Council for Health Research and Development, Department of Science and Technology, Republic of the Philippines and administered through the University of the Philippines Manila. This study was supported by 1R01HD100729 (NIH/NICHD) to Dr. Ramkumar Menon.

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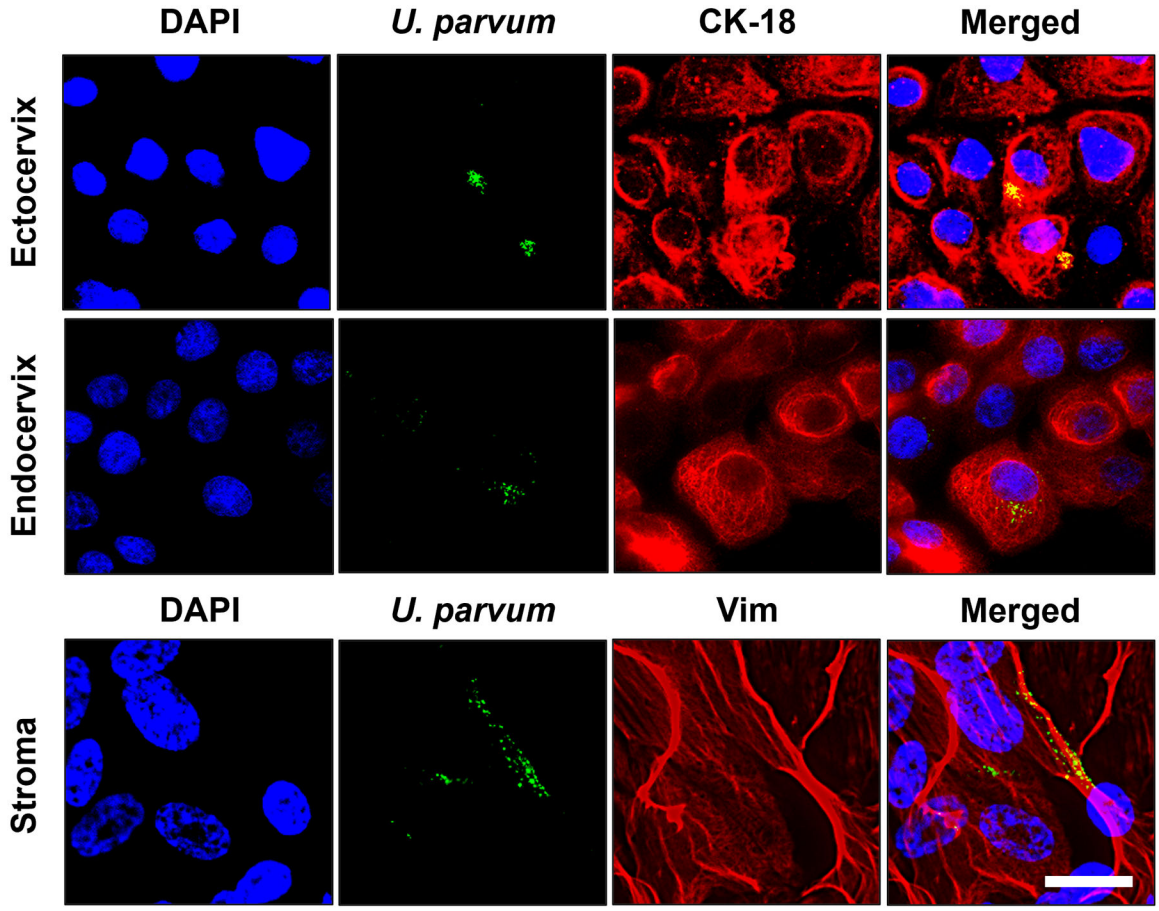

Fig 1. *U. parvum* can colonize cervical epithelial and stromal cells.

Fluorescence microscopy imaging showing DiO-labeled *U. parvum* (green) in human cervical epithelial and stromal cells 4 h post-infection. CK-18 (red) was used as a marker for ectocervical and endocervical epithelial cells while vimentin (red) was used as marker for cervical stromal cells. Nuclei are stained with DAPI, n=3 technical replicates. Scale bar, 25 μm.

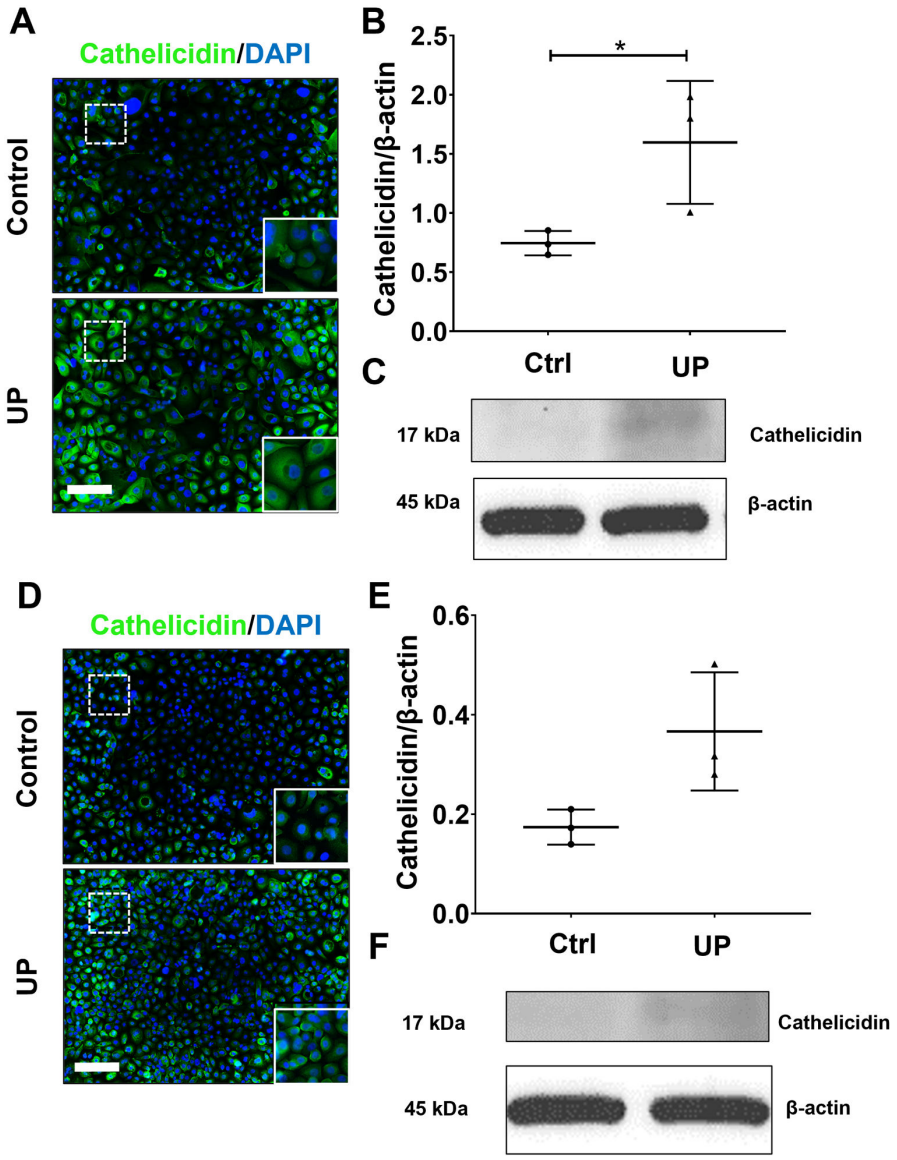

Fig 2. *U. parvum* increases antimicrobial peptide production in cervical epithelilal cells.

Fluorescence microscopy imaging and western blot analyses showing cathelicidin in uninfected and *U. parvum*-infected human ectocervical (**A – C**) and endocervical epithelial cells (**D – F**). Nuclei are stained with DAPI, n=3 technical replicates. Scale bar, 100 μm. Western blot analysis and quantification of cathelicidin in ectocervical (**B – C**) and endocervical epithelial cells (**E – F**). β-actin is used as loading control. Error bars represent mean ± SEM, n=3 technical replicates. Linear adjustment of contrast and brightness has been applied to all fluorescent images throughout the figure. *, $p < 0.05$.

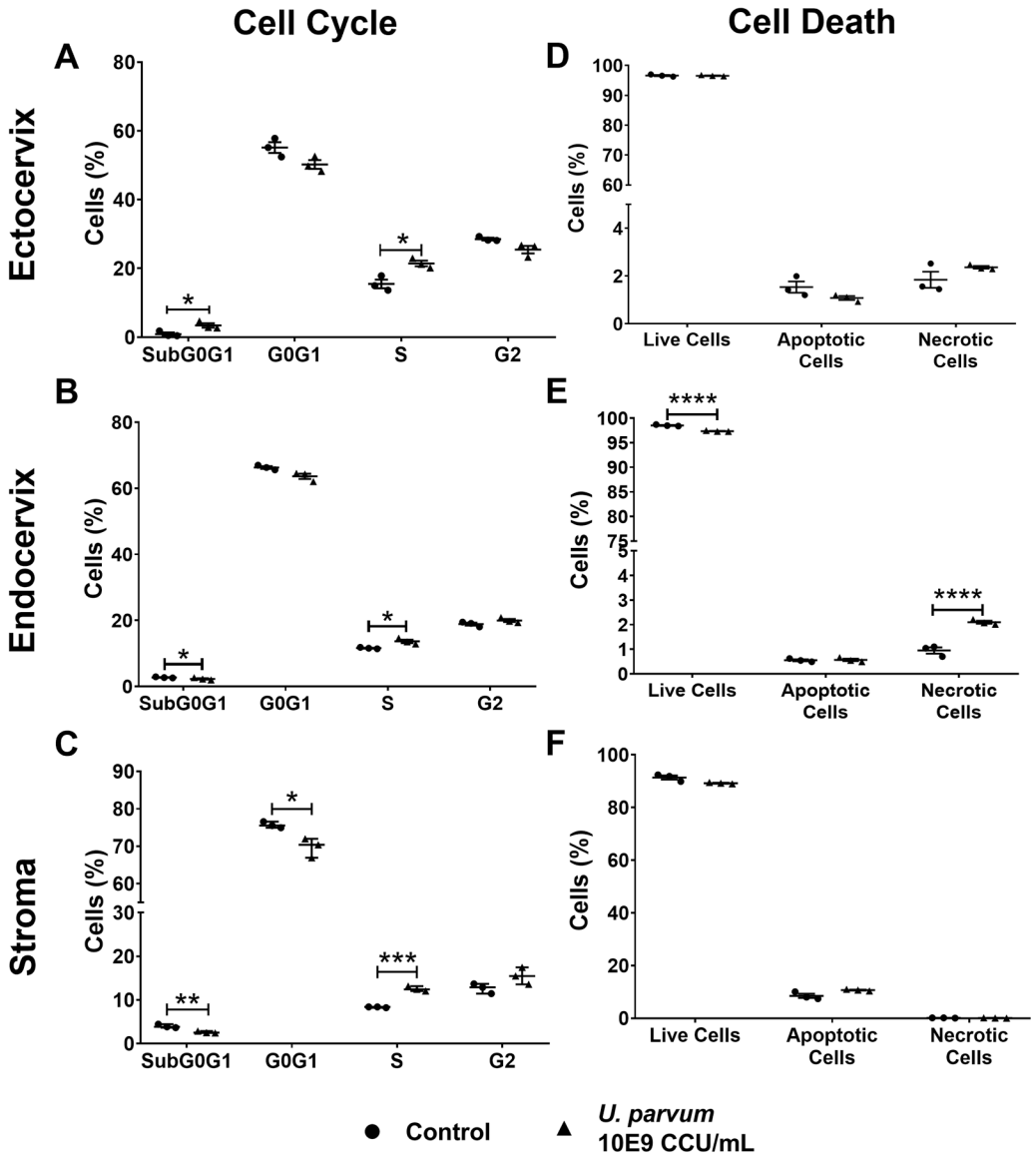

Fig 3. *U. parvum* increases G1-S phase transition and does not promote cell death in cervical epithelial and stromal cells.

Quantification of flow cytometric analysis of cell cycle progression of ectocervical (**A**) and endocervical epithelial cells (**B**), and cervical stromal cells (**C**) after 48 h of *U. parvum* infection. Error bars represent mean % cells ± SEM, n=3 technical replicates. Flow cytometric analysis of Annexin V/PI-stained, apoptotic, and necrotic ectocervical (**D**) and endocervical epithelial cells (**E**), and cervical stromal cells (**F**). Error bars represent mean % cells ± SEM (A, B, D, E, and F) or median % cells ± SD (C), n=3 technical replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

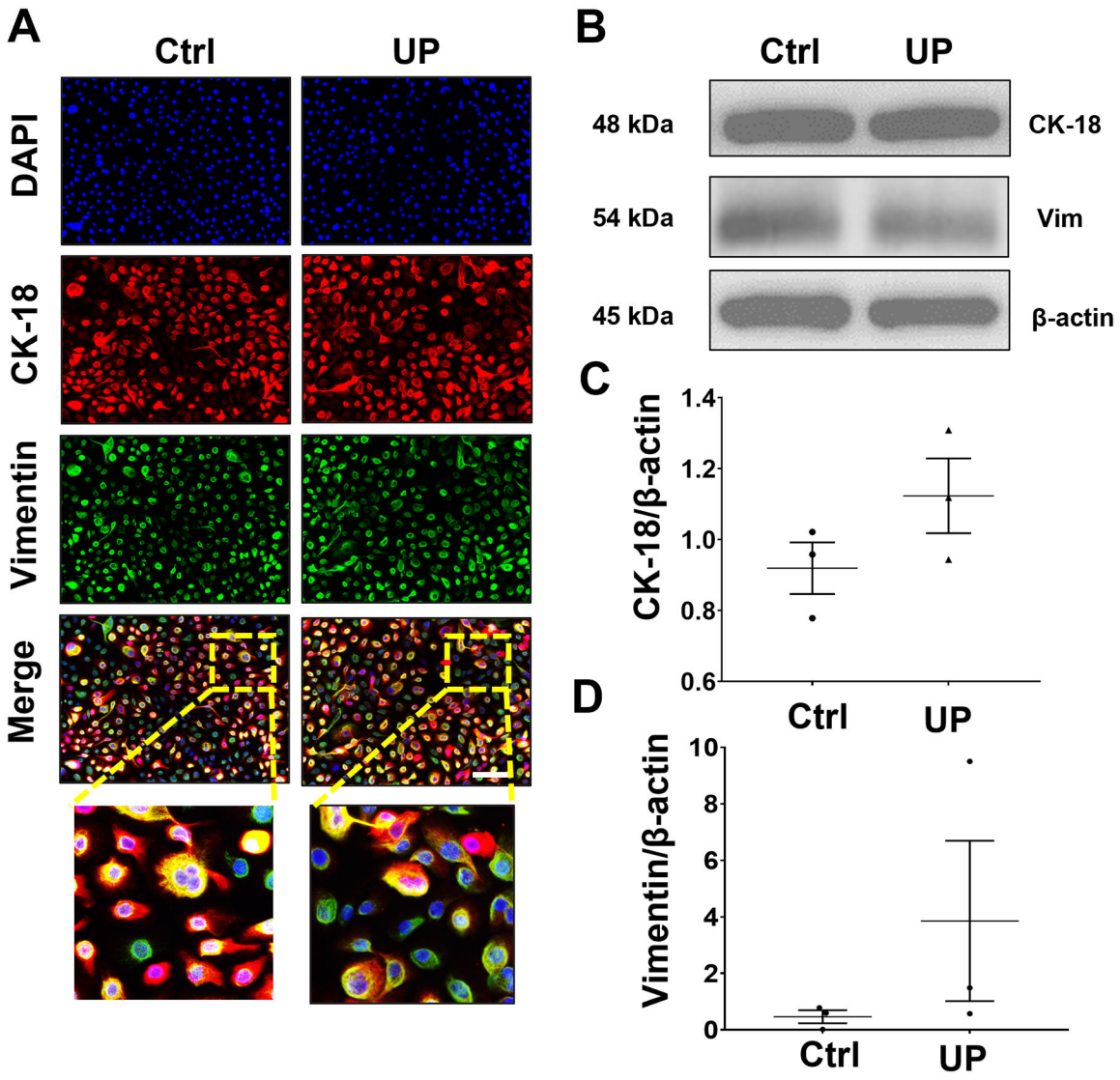

Fig 4. *U. parvum* promotes EMT in ectocervical epithelial cells.

(**A**) Fluorescence microscopy imaging showing CK-18 and vimentin in uninfected and *U. parvum*-infected human ectocervical epithelial cells. Nuclei are stained with DAPI, n=3 technical replicates. Scale bar, 100 μm. Western blot analysis and quantification of CK-18 (**B, C**) and vimentin (**B, D**) in ectocervical epithelial cells. β-actin is a loading control. Error bars represent mean ± SEM, n=3 technical replicates. Linear adjustment of contrast and brightness has been applied to all fluorescent images throughout the figure. *, $p < 0.05$.

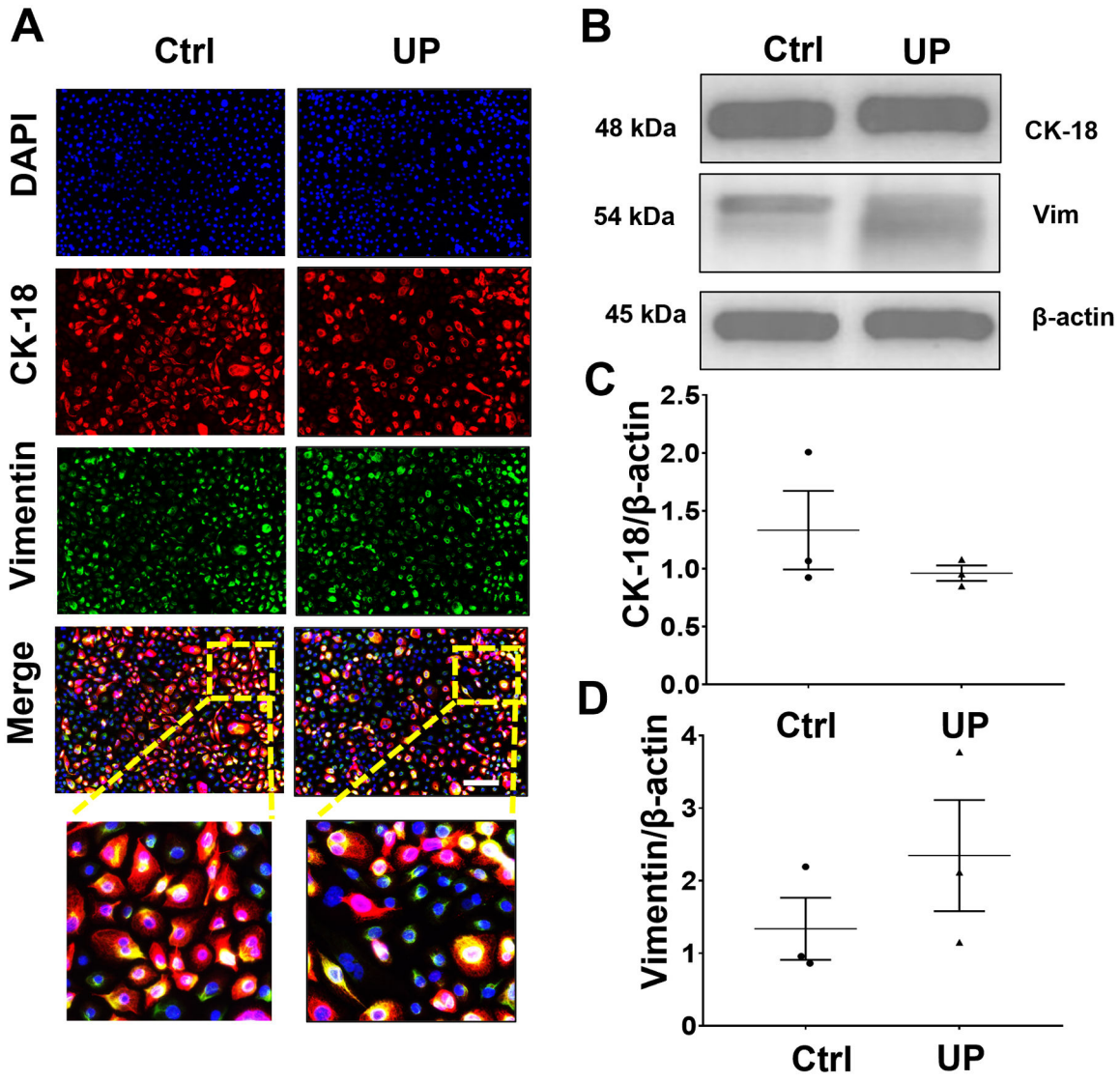

Fig 5. *U. parvum* promotes EMT in endocervical epithelial cells.

(**A**) Fluorescence microscopy imaging showing CK-18 and vimentin in uninfected and *U. parvum*-infected human endocervical epithelial cells. Nuclei are stained with DAPI, n=3 technical replicates. Scale bar, 100 μm. Western blot analysis and quantification of CK-18 (**B, C**) and vimentin (**B, D**) in endocervical epithelial cells. β-actin is a loading control. Error bars represent mean ± SEM, n=3 technical replicates. Linear adjustment of contrast and brightness has been applied to all fluorescent images throughout the figure.

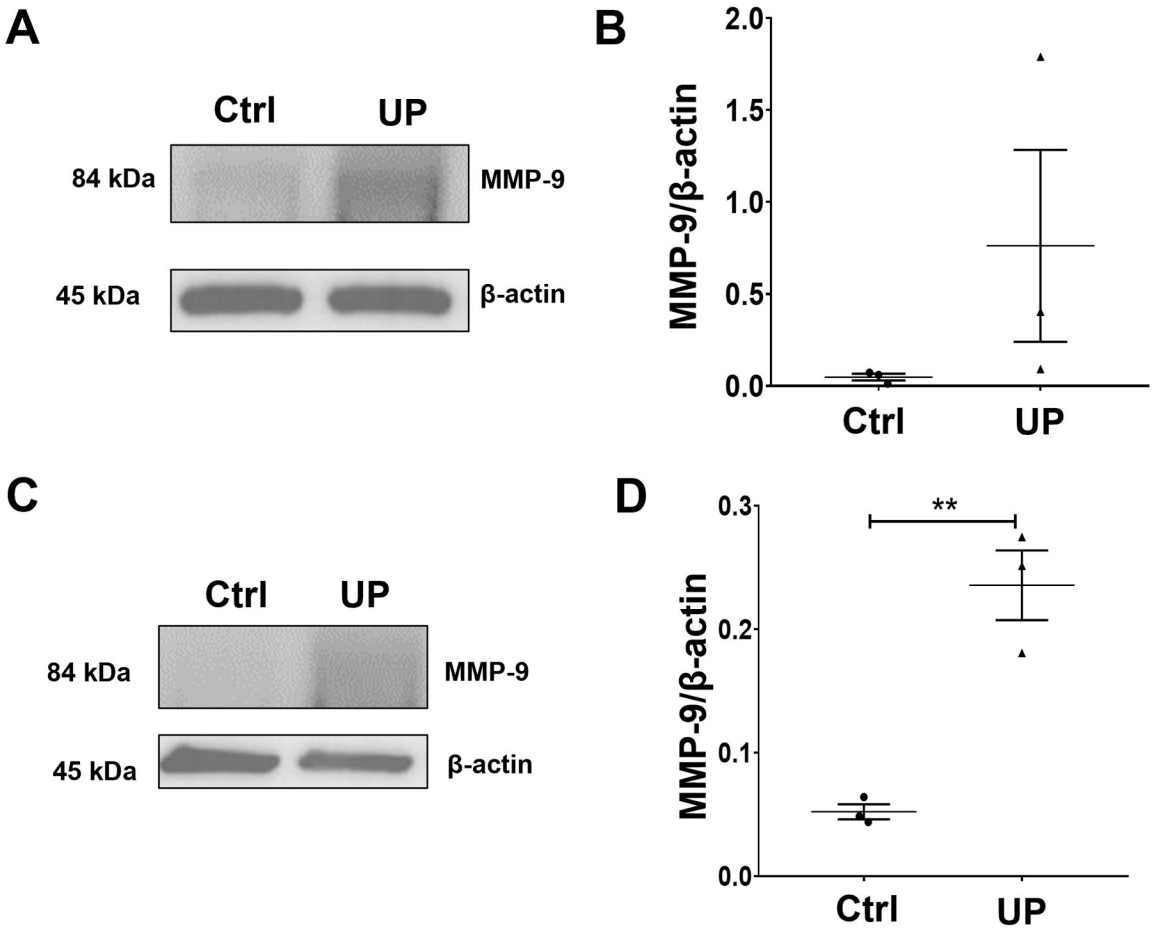

Fig 6. *U. parvum* increases MMP-9 in cervical epithelial cells.

Western blot analysis and quantification of MMP-9 in ectocervical (**A, B**) and endocervical epithelial cells (**C, D**). β-actin is a loading control. Error bars represent mean ± SEM, n=3 technical replicates. **, $p < 0.01$.

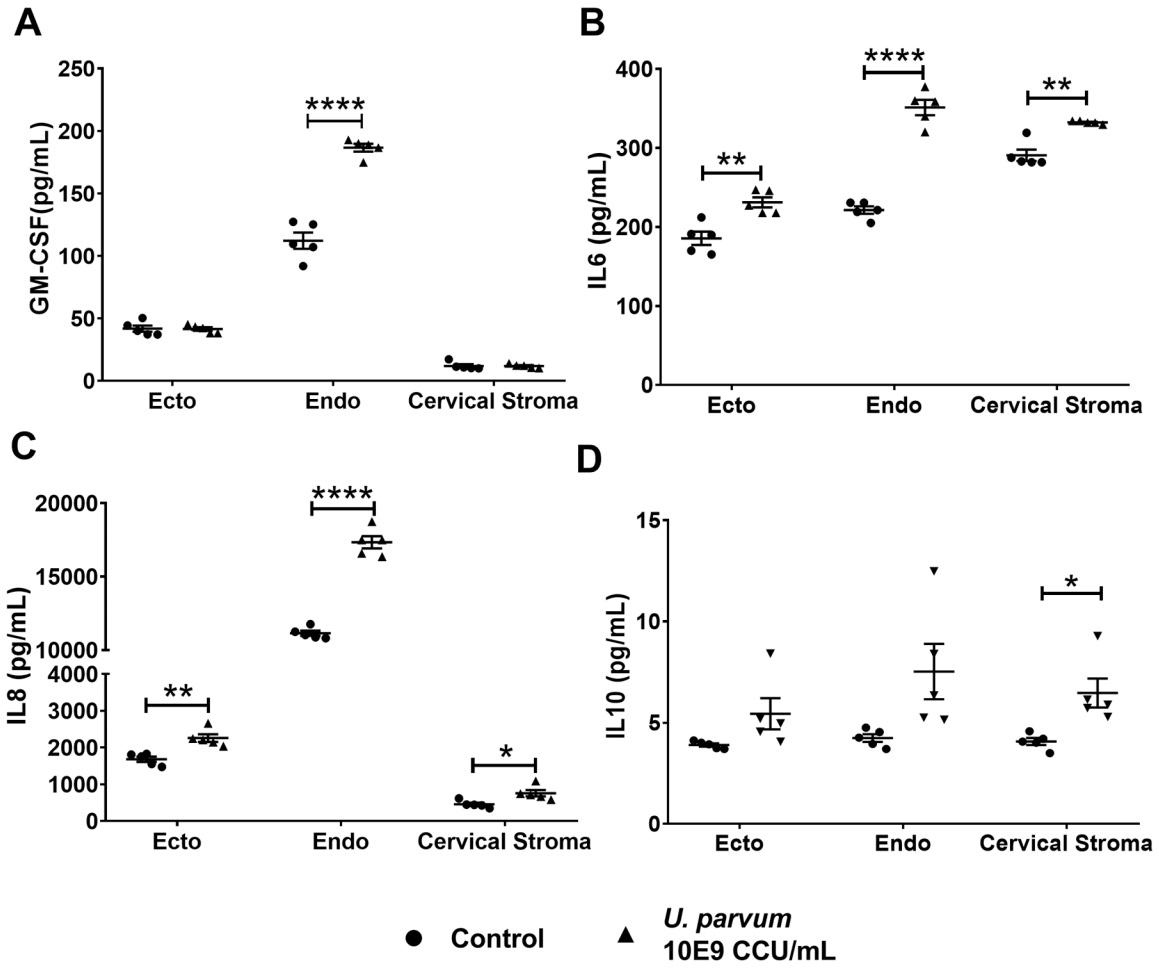

Fig 7. *U. parvum* promotes differential inflammatory responses in cervical epithelial and stromal cells.

Pro-inflammatory cytokines, human GM-CSF (**A**), IL-6 (**B**), and IL-8 (**C**), and anti-inflammatory cytokine IL-10 (**D**) levels in culture medium collected from ectocervical and endocervical epithelial cells, and cervical stromal cells after 48 h of *U. parvum* infection. Error bars represent mean concentration ± SEM, n=5 technical replicates. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

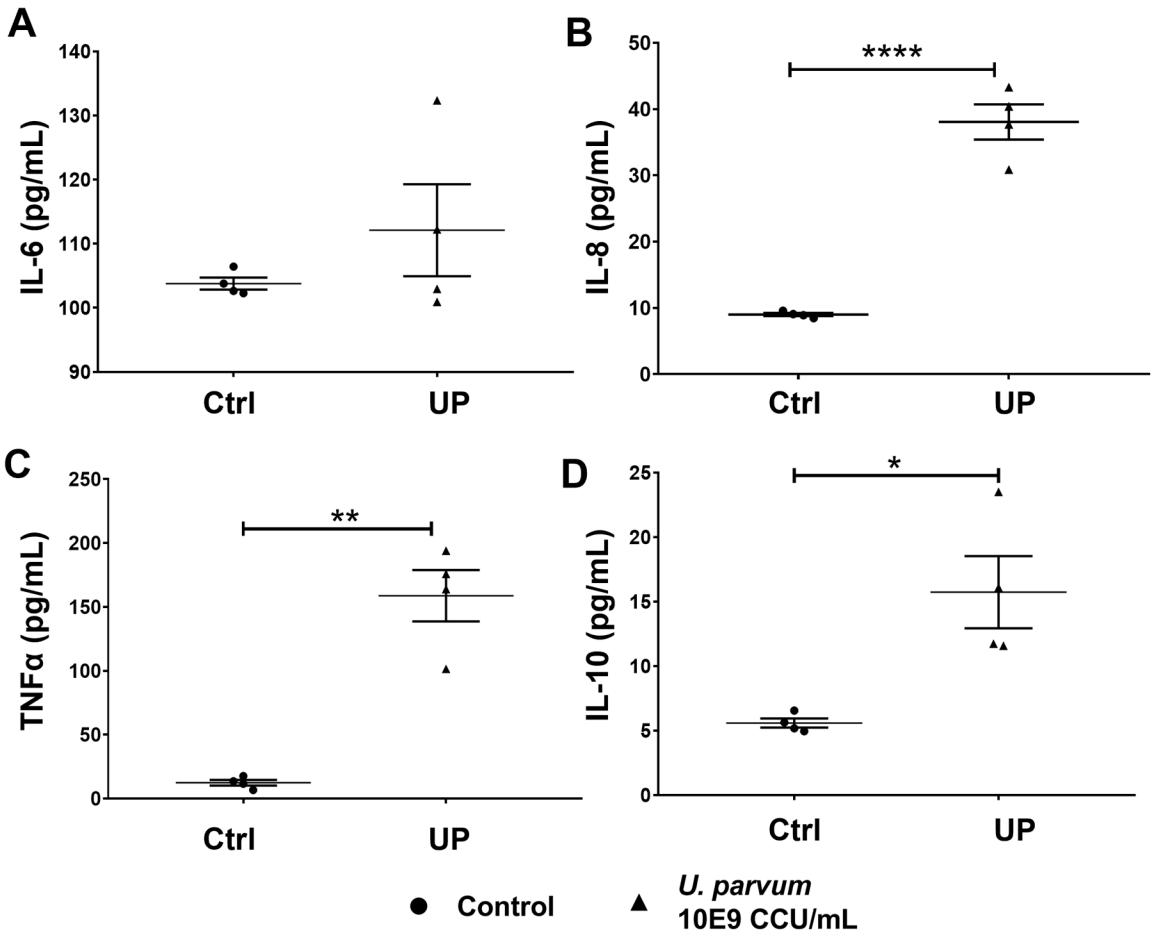

Fig 8. *U. parvum* infection promotes both pro- and anti-inflammatory responses in human macrophages.

Pro-inflammatory cytokines human IL-6 (**A**), IL-8 (**B**), and TNFα (**C**), and anti-inflammatory cytokine IL-10 (**D**) levels in culture medium collected from THP-1 macrophages after 48 h of *U. parvum* infection. Error bars represent mean concentration ± SEM, n=5 technical replicates. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.