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Amniotic fluid neutrophils can phagocytize bacteria: A mechanism for microbial killing in the amniotic cavity

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Problem: Neutrophils are capable of performing phagocytosis, a primary mechanism for microbial killing. Intra-amniotic infection is characterized by an influx of neutrophils into the amniotic cavity. Herein, we investigated whether amniotic fluid neutrophils could phagocytize bacteria found in the amniotic cavity of women with intra-amniotic infection.

Methods: Amniotic fluid neutrophils from women with intra-amniotic infection were visualized by transmission electron microscopy (n=6). The phagocytic activity of amniotic fluid neutrophils from women with intra-amniotic infection and/or inflammation (n=10) or peripheral neutrophils from healthy individuals (controls, n=3) was tested using ex vivo phagocytosis assays coupled with live imaging. Phagocytosis by amniotic fluid neutrophils was also visualized by confocal microscopy (n=10) as well as scanning and transmission electron microscopy (n=5).

Results: (i) Intra-amniotic infection-related bacteria including cocci (eg *Streptococcus agalactiae*), bacilli (eg *Bacteriodes fragilis* and *Prevotella* spp.), and small bacteria without a cell wall (eg *Ureaplasma urealyticum*) were found inside of amniotic fluid neutrophils; (ii) peripheral neutrophils (controls) rapidly phagocytized *S. agalactiae*, *U. urealyticum*, *Gardnerella vaginalis*, and *Escherichia coli*; (iii) amniotic fluid neutrophils rapidly phagocytized *S. agalactiae* and *G. vaginalis*; and (iv) amniotic fluid neutrophils slowly phagocytized *U. urealyticum* and *E. coli*; yet, the process of phagocytosis of the genital mycoplasma was lengthier.

Conclusion: Amniotic fluid neutrophils can phagocytize bacteria found in the amniotic cavity of women with intra-amniotic infection, namely *S. agalactiae*, *U. urealyticum*, *G. vaginalis*, and *E. coli*. Yet, differences in the rapidity of phagocytosis were observed among the studied microorganisms. These findings provide a host defense mechanism whereby amniotic fluid neutrophils can kill microbes invading the amniotic cavity.

KEYWORDS
acute chorioamnionitis, clinical chorioamnionitis, cytokine, fetal inflammatory response, fever, funisitis, human, inflammation, innate immune cells, interleukin-6, intra-amniotic infection, labor, microbial invasion of the amniotic cavity, parturition, phagocytosis, pregnancy, preterm birth, preterm labor

1 | INTRODUCTION

Intra-amniotic infection is a clinical condition characterized by a local inflammatory process caused by microbial invasion of the amniotic cavity (MIAC).[1-9] Microorganisms associated with intra-amniotic infection are commonly found in the lower genital track, including *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae* (also referred to as Group B Streptococcus or GBS), *Gardnerella vaginalis*, and *Escherichia coli*, among others.[10-14] This inflammatory response can result in systemic[10,15-18] and/or local[19-25] inflammatory responses. Systemically, intra-amniotic infection can be manifested as clinical chorioamnionitis, which refers to the presence of maternal fever associated with clinical signs (foul-smelling discharge and uterine tenderness as well as maternal and fetal tachycardia) and laboratory abnormalities such as leukocytosis.[10,15-18] Locally, intra-amniotic infection is characterized by an increased white blood cell (WBC) count[26-31] and elevated concentrations of cytokines[8,32] and lipid mediators (eg prostaglandins)[33-47] in the amniotic cavity.

The most abundant WBCs (ie leukocytes) in the amniotic cavity of women with intra-amniotic infection are the neutrophils; therefore, their number is a useful marker for the detection of intra-amniotic inflammation.[26,31] Amniotic fluid neutrophils are a part of the innate immune host defense mechanisms that take place in the amniotic cavity of women with intra-amniotic infection.[48-50] Indeed, amniotic fluid neutrophils are a source of antimicrobial products[51-55] and cytokines.[31] In addition, these innate immune cells can trap and kill bacteria invading the amniotic cavity by forming neutrophil extracellular traps or NETs.[56] Neutrophils infiltrating the chorioamniotic membranes also form NETs in cases with acute histologic chorioamnionitis,[57] a placental lesion associated with elevated concentrations of pro-inflammatory cytokines in the amniotic fluid.[32,48,58-75] The formation of NETs or NETosis[76] represents the final containment effort of a neutrophil to kill pathogens.[77] NETs are web-like structures composed of DNA, histones, and antimicrobial products that trap and/or eliminate microbes through their biochemical components.[77-80] Yet, only a fraction (~20%) of human neutrophils,[81] including those in the amniotic cavity,[56] form NETs. This suggests that, in addition to forming NETs, amniotic fluid neutrophils use other host defense mechanisms against microorganisms invading the amniotic cavity.

Neutrophils are primarily capable of performing phagocytosis,[82-84] a main mechanism for microbial killing.[85] Phagocytosis is the receptor-mediated process whereby a cell (eg neutrophil) extends its plasma membrane around the target (eg microbe), initiating the formation of a membrane-bound vacuole termed the phagosome.[86,87] Such a phagosome requires a process of maturation, which comprises the acquisition of microbicidal enzymes, vacuolar ATPases, and the NADPH oxidase complex.[86] In neutrophils, however, the process of phagosome maturation seems to start even before microbe ingestion, indicating that the content, membrane composition, pH, and signaling in the phagosome are different from those made by other phagocytes (eg macrophages).[87] The antimicrobial effect of the neutrophil phagosome is due to the fusion of its granules with secretory vesicles, which contain albumin and express alkaline phosphatase and CD35 on their membranes.[86] Neutrophils contain three types of cytoplasmic granules: (i) primary (or azurophilic) granules, which are positive for peroxidase and have lytic enzymes and defensins; (ii) secondary granules (or specific granules), which contain lactoferrin; and (iii) tertiary or gelatinase granules.[86,87] The fusion of granule components with phagosomes and/or the plasma membrane is orchestrated by the NADPH oxidase complex, generating reactive oxygen species (ROS).[87] The timing and execution of this process must be carefully regulated to kill microbes without causing tissue damage to the host.

As neutrophil phagocytosis is a main host defense mechanism for microbial killing, we investigated whether: (i) intra-amniotic infection-related bacteria were found engulfed in amniotic fluid neutrophils using transmission electron microscopy; and (ii) amniotic fluid neutrophils could phagocytize bacteria associated with intra-amniotic infection (*S. agalactiae*, *U. urealyticum*, *G. vaginalis*, and *E. coli*) in a similar manner to peripheral neutrophils by using ex vivo phagocytosis assays coupled with live imaging. Phagocytosis by amniotic fluid neutrophils was also visualized by confocal microscopy as well as scanning and transmission electron microscopy.

2 | MATERIALS AND METHODS

2.1 | Study population

This was a cross-sectional study of patients who underwent transabdominal amniocentesis due to clinical indications or amniocentesis during cesarean section. Patients were enrolled at Hutzel Women's Hospital of the Detroit Medical Center (November 2015 to November 2016). The initial observation of in vivo phagocytosis (amniotic fluid neutrophils with engulfed bacteria) was made using transmission electron microscopy in 6 amniotic fluid samples from women diagnosed with intra-amniotic infection (Table 1; see below for clinical definitions). For ex vivo phagocytosis assays, 10 amniotic fluid samples were collected from women with suspected intra-amniotic infection and/or inflammation (Table 2; see below for clinical definitions) and were immediately transported to the clinical and research laboratories. All of the amniotic fluid samples were acquired by an automatic cell counter (Cellometer Auto 2000; Nexcelom Bioscience, Lawrence, MA, USA) to obtain the viable cell numbers. Most of the viable cells are leukocytes.[31] The inclusion criteria were (i) singleton gestations, (ii) samples without blood contamination, and (iii) sufficient amniotic fluid leukocytes (>1×10^5 cells/mL) to evaluate in vivo phagocytosis using transmission electron microscopy or to perform ex vivo phagocytosis assays coupled with live imaging, confocal microscopy, and scanning and transmission electron microscopy.

All of the patients provided written informed consent to donate additional amniotic fluid for research purposes, according to protocols approved by the Institutional Review Boards of the Detroit Medical Center (Detroit, MI, USA), Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services (NICHD/NIH/DHHS).

2.2 | Clinical definitions

Gestational age was determined by the last menstrual period and confirmed by ultrasound examination. The gestational age derived from sonographic fetal biometry was used when the estimation was inconsistent with menstrual dating. Clinical chorioamnionitis was diagnosed by the presence of maternal fever (temperature >37.8°C) accompanied by two or more of the following criteria: (i) uterine tenderness; (ii) malodorous vaginal discharge; (iii) fetal tachycardia (heart rate >160 beats/min); (iv) maternal tachycardia (heart rate >100 beats/min); and (v) maternal leukocytosis (leukocyte count >15 000 cells/mm^3). Term delivery was defined as birth after 37 weeks of gestation, whereas preterm delivery was defined as birth between 20 and 36 6/7 weeks of gestation.

Microbial invasion of the amniotic cavity was defined as a positive amniotic fluid culture.[11,64,65,88-90] Intra-amniotic inflammation was diagnosed when the interleukin (IL)-6 concentration in amniotic fluid was ≥2.6 ng/mL.[91] Intra-amniotic infection was defined as the presence of MIAC with intra-amniotic inflammation.[12,13,91-104]

2.3 | Placental histopathological examination

Five-μm-thick sections of formalin-fixed, paraffin-embedded tissue specimens were cut and mounted on SuperFrost™ Plus microscope slides (Erie Scientific LLC, Portsmouth, NH, USA). In each case, several tissue sections of the chorioamniotic membranes, umbilical cord, and placental disk were examined. After deparaffinization, slides were rehydrated, stained with hematoxylin-eosin, and evaluated by pathologists who had been blinded to the clinical outcome. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to protocols from the Perinatology Research Branch. While the stage of the placental lesion refers to the progression of the inflammatory process based on the anatomical regions infiltrated by neutrophils (stage 1-3), the grade of the placental lesion is defined by the intensity of the acute inflammatory process at a particular site [grade 1 (mild to moderate) and grade 2 (severe)]. For more information about the staging and grading of the acute inflammatory lesions of the placenta, please see placental pathology reviews.[105-108]

2.4 | Sample collection

Amniotic fluid was retrieved by transabdominal amniocentesis under antiseptic conditions using a 22-gauge needle monitored by ultrasound. Amniotic fluid was also retrieved by amniocentesis during cesarean section under antiseptic conditions. Amniotic fluid samples were transported to the clinical laboratory in a capped sterile syringe and were cultured for aerobic and anaerobic bacteria as well as for genital mycoplasmas.[13,26,109-112] Shortly after collection, the WBC count in amniotic fluid samples was determined by using a hemocytometer chamber, according to methods previously described.[26] Glucose concentration was also determined,[113] and Gram stain[114] was performed in amniotic fluid samples. Cultures, WBC count, glucose concentration, and Gram stain were not performed in all of the amniotic fluid samples collected during cesarean section, as these samples were collected for research purposes only. However, both IL-6 concentration and the presence of bacteria (bacterial live/dead staining[56,115]) were assessed in most of the amniotic fluid samples.

2.5 | Determination of interleukin-6 in the amniotic fluid

IL-6 concentrations in the amniotic fluid were determined using a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, MN, USA). The IL-6 concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL.

2.6 | Detection of live/dead bacteria in the amniotic fluid

The presence of bacteria in the amniotic fluid was evaluated as previously described,[56,115] using the LIVE/DEAD BacLight™ Bacterial Viability Kit (Cat# L7007; Life Technologies, Grand Island, NY, USA) in a sterile biosafety cabinet. Briefly, 100 μL of amniotic fluid were mixed with 900 μL of sterile 1X phosphate-buffered saline (PBS; Life Technologies). Three microliters of the dye mix (components A and B were mixed at a 1:1 ratio) were added to the cell suspension and incubated for 15 minutes at room temperature in the dark. Next, the cells were centrifuged at 10 000 *g* for 5 minutes and the supernatant was discarded. The cell pellet was then re-suspended in 5 μL of 1X PBS, and a slide smear was prepared and air-dried. Lastly, the slide was gently rinsed with 1X PBS and mounted with ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole or DAPI (Life Technologies). The presence of bacteria was evaluated using an Olympus BX 60 fluorescence microscope with an Olympus DP71 camera and DP Controller Software (Olympus Corporation, Tokyo, Japan).

2.7 | Transmission electron microscopy of in vivo phagocytosis

Amniotic fluid samples (Table 1) were passed through a sterile 15-μm filter (Cat# 43-50015-03, pluriSelect Life Science; Leipzig, Germany) and centrifuged at 2300 *g* for 5 minutes at room temperature, and the supernatant was discarded. Electron microscopy fixative (2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4; Cat# 16537-05, Electron Microscopy Science, Hatfield, PA, USA) was carefully added to the cell pellet. Following fixation for 2 hours at 4°C, the cell pellet was gently washed with 1X electron microscopy wash buffer (Sorensen's phosphate buffer 0.2 mol/L, pH 7.4; Cat# 11601-10, Electron Microscopy Science). Cell pellets from amniotic fluid samples were transported to the Microscopy & Image Analysis Laboratory at the University of Michigan (https://medicine.umich.edu/medschool/research/office-research/biomedical-research-core-facilities/

TABLE 1 Clinical characteristics of amniotic fluid samples in which in vivo phagocytosis was observed

Sample	Clinical chorioamnionitis	Viable cell count[a] (cells/mm^3)	Gestational age at amniocentesis	Collection method for amniotic fluid	IL-6 (ng/mL)	Gram stain	Bacterial live/dead staining	Amniotic fluid culture	WBC (cells/mm^3)	Glucose (mg/dL)	Gestational age at delivery	Placental pathology	
												Acute maternal inflammatory response	Acute fetal inflammatory response
1	Yes	2200	36.6	Transabdominal	8.1	Gram-positive cocci	Positive	*Streptococcus agalactiae*	310	<1	36.7	Stage 3	Stage 2
2	No	100	18.9	Transabdominal	121.3	Gram-negative bacilli	Positive	*Bacteroides fragilis*	65	20	19.6	Stage 3	Stage 2
3	Yes	18 800	40	C/S	47.6	Negative	Positive	*Ureaplasma urealyticum*	NA	NA	40	Stage 2	Stage 2
4	No	9920	23	Transabdominal	27	Gram-positive cocci, Few Gram-negative coccobacilli	Positive	*Enterobacter aerogenes*, *Enterococcus faecalis*, *Mycoplasma hominis*, *Prevotella* spp., *Streptococcus viridans*	6938	4	25.7	Stage 3	Stage 1
5	Yes	2200	35.6	Transabdominal	70.6	Gram-positive cocci, Few Gram-positive bacilli and Gram-negative bacilli	Positive	*Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Prevotella* spp.	4000	<1	35.6	Stage 2	Stage 3
6	Yes	6780	35.6	C/S	NA	Gram-positive cocci, Gram-negative bacilli	Positive	*Ureaplasma urealyticum*, *Mycoplasma hominis*, *Prevotella* spp.	7920	<1	35.6	Stage 2	Stage 3

CS, cesarean section; IL, interleukin; NA, not available; WBC, white blood cell.
[a]Viable cell count: Determined with AO/PI on Cellometer 2000 Auto (Nexcelom).

TABLE 2 Clinical characteristics of amniotic fluid samples utilized for ex vivo phagocytosis assays

Sample	Clinical chorioamnionitis	Viable cell count[a] (cells/mm^3)	Gestational age at amniocentesis	Collection method for amniotic fluid	IL-6 (ng/mL)	Gram stain	Bacterial live/dead staining	Amniotic fluid culture	WBC (cells/mm^3)	Glucose (mg/dL)	Gestational age at delivery	Placental pathology	
												Acute maternal inflammatory response	Acute fetal inflammatory response
1	No	100	18.9	Transabdominal	121.3	Gram-negative bacilli	Positive	*Bacteroides fragilis*	65	20	19.6	Stage 3	Stage 2
2	Yes	2200	35.6	Transabdominal	70.6	Gram-positive cocci, Few Gram-positive bacilli and Gram-negative bacilli	Positive	*Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Prevotella* spp.	4000	<1	35.6	Stage 2	Stage 3
3	No	3660	21.3	Transabdominal	118.7	Negative	Negative	*Staphylococcus hominis*	355	<1	21.9	Stage 3	Stage 2
4	No	1160	22.3	Transabdominal	125.5	Gram-negative bacilli	Positive	*Mycoplasma hominis*, *Fusobacterium nucleatum*	700	10	22.7	Stage 2	Stage 1
5	Yes	860	39.9	Transabdominal	73.5	Negative	Negative	Negative	600	<1	40	Stage 2	Stage 2
6	Yes	535	39.6	Transabdominal	3.5	Negative	Positive	*Mycoplasma hominis*, *Ureaplasma urealyticum*	590	<1	39.6	Stage 1	Stage 1
7	No	258	40.6	C/S	1.9	Negative	Negative	*Ureaplasma urealyticum*, *Staphylococcus haemolyticus*	NA	NA	40.6	None	None
8	Yes	18 800	40	C/S	47.6	Negative	Positive	*Ureaplasma urealyticum*	NA	NA	40	Stage 2	Stage 2
9	No	9600	38.1	C/S	101.3	NA	Positive	*Mycoplasma hominis*, *Ureaplasma urealyticum*	NA	NA	38.1	Stage 1	Stage 2
10	No	286	39.3	Transabdominal	0.5	NA	Negative	Negative	NA	NA	39.3	None	None

CS, cesarean section; IL, interleukin; NA, not available; WBC, white blood cell.

[a]Viable cell count: Determined with AO/PI on Cellometer 2000 Auto (Nexcelom).

microscopy-image-analysis). Images were obtained using a JEOL JSM-1400 plus transmission electron microscope (JEOL, Peabody, MA, USA).

2.8 | Bacteria strains and growth conditions for ex vivo phagocytosis assays

Streptococcus agalactiae (ATCC® 13813), *U. urealyticum* (ATCC® 27618), *G. vaginalis* (ATCC® 14018), and *E. coli* (*E. coli*, ATCC® 700926) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). *Ureaplasma urealyticum* was also isolated from a patient with intra-amniotic infection. *Streptococcus agalactiae* and *G. vaginalis* were cultured in brain-heart infusion broth (BHI, Cat#R060260, Remel, Lenexa, KS, USA) at 37°C with shaking at 180 rpm. *Escherichia coli* was grown in Luria-Bertani broth (LB, Cat#L7658, Sigma, Saint Louis, MO, USA) at 37°C with shaking at 180 rpm. An overnight culture was diluted into fresh medium and grown to the mid-logarithmic phase (OD_{600} was between 0.5 and 1.0). Bacteria were then harvested by centrifugation at 2300 *g* for 5 minutes and re-suspended in 1X PBS. *Ureaplasma urealyticum* obtained from ATCC or a clinical sample was cultured in SP4 Broth with urea (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C with shaking at 180 rpm until a color change (yellow to pink) was observed. The culture broth was then centrifuged at 1500 *g* for 30 minutes at 4°C. The identification of characteristic colonies of *U. urealyticum* was performed on an A8 agar plate (Hardy Diagnostics).

2.9 | Fluorescent labeling of bacteria for ex vivo phagocytosis assays

Heat-killed bacteria were labeled using the Alexa Fluor® 488 Antibody Labeling Kit (CAT# A20181, Life Technologies). Briefly, heat-killed bacteria were re-suspended in 1X PBS and sodium bicarbonate solution was added to a final concentration of 0.1 mol/L. This solution was then added to a vial of Alexa Fluor® 488 dye and incubated for 1 hour at room temperature in the dark. Bacteria were then centrifuged, washed, and re-suspended in 1X PBS containing 20% glycerol (Cat#G1796, TEKnova, Hollister, CA, USA) to an OD_{600} of 0.3 (~1.5×10^7/50 µL). Fluorescent-labeled bacteria were aliquoted and stored in −80°C until use.

2.10 | Opsonization of bacteria for ex vivo phagocytosis assays

Fluorescent-labeled bacteria were thawed and incubated with heat-inactivated-pooled human serum (Cat#1830-0002, Sera Care, Milford, MA, USA) for 30 minutes at 37°C with a gentle rotation. Bacteria were washed with 1X PBS and re-suspended in RPMI-1640 culture medium supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies; hereafter referred to as "supplemented RPMI medium") for ex vivo phagocytosis assays coupled with live imaging, confocal microscopy, and scanning and transmission electron microscopy.

2.11 | Live imaging of ex vivo phagocytosis assays

Amniotic fluid samples (Table 2) were passed through a sterile 15-µm filter and centrifuged at 200 *g* for 5 minutes at room temperature. This step allows the enrichment of amniotic fluid leukocytes (mostly neutrophils[31]) and the elimination of epithelial cells.[56] Amniotic fluid leukocytes were then re-suspended in supplemented RPMI medium at a concentration of 2.5×10^5 cells/0.5 mL, plated in a 35-mm culture dish with a cover glass bottom (MatTek, Ashland, MA, USA), and labeled with an anti-human CD15-PE-CF594 antibody (Clone W6D3, Cat#562372, BD Biosciences, San Jose, CA, USA). Following 15 minutes of incubation at 37°C, amniotic fluid leukocytes were gently washed with supplemented RPMI medium. Amniotic fluid neutrophils were visualized on a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at the Microscopy, Imaging, and Cytometry Resources Core at the Wayne State University School of Medicine (http://micr.med.wayne.edu/), using a W Plan-Apochromat 63X/1.0 objective, which was immersed in the cell culture with supplemented RPMI medium. Live imaging of ex vivo phagocytosis assays was performed after adding 50 µL of the opsonized fluorescent-labeled bacteria to the culture plate. Confocal time series of amniotic fluid neutrophils phagocytizing bacteria were recorded with a frame size of 512×512 pixels at 7.75-second time intervals from 5 to 20 minutes. A semi-quantification of the time-interval for each ex vivo phagocytosis assay was calculated based on the duration of the assay and the number of frames taken per experiment.

As controls, peripheral neutrophils were isolated from healthy individuals (n=3) using the density gradient reagent Histopaque 1119 (Sigma-Aldrich), according to the manufacturer's instructions and a previously published method.[116] Briefly, 6 mL of peripheral blood was layered on top of 6 mL of Histopaque 1119 and centrifuged at 800 *g* for 20 minutes with no break at room temperature. Neutrophils were collected from the lower phase of the gradient after the peripheral blood mononuclear cell band was discarded. The collected neutrophils were further purified using a gradient composed of 85%, 80%, 75%, 70%, and 65% Percoll (GE Healthcare Life Sciences; Uppsala, Sweden) and washed with 1X PBS. Purified neutrophils were then incubated with labeled bacteria for ex vivo phagocytosis assays, and a semi-quantification was performed as described above.

2.12 | Confocal microscopy of ex vivo phagocytosis assays

Neutrophils were enriched from amniotic fluid samples (Table 2), as described above, and placed in a 24-well culture plate (Corning Life Sciences, Durham, NC, USA) containing 12-mm cover slips (Fisher Scientific, Waltham, MA, USA) at a concentration of 2.5×10^5 cells/0.5 mL for 1 hour at 37°C in supplemented RPMI medium. Following the attachment of neutrophils to the cover slips, medium was replaced with 200 µL of fresh medium and 20 µL of an anti-human CD15-PE-CF594 antibody were added to the culture dish. After 30 minutes of incubation, excess antibody was removed by gently washing

the amniotic fluid neutrophils with supplemented RPMI medium. Next, 500 μL of fresh medium and 50 μL of opsonized fluorescent-labeled bacteria were added to the amniotic fluid neutrophils. The culture plate was then centrifuged at 600 *g* for 4 minutes and incubated for 1 hour at 37°C for ex vivo phagocytosis assays. Following incubation, amniotic fluid neutrophils were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Science) and the cover slips were carefully removed from the culture plate. Lastly, the cover slips were mounted onto Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, Wilmington, DE, USA) using ProLong Diamond Antifade Mountant with DAPI. Amniotic fluid neutrophils containing phagocytized bacteria were visualized on a Zeiss LSM 780 laser scanning confocal microscope. Confocal z-stacks were acquired using a Plan-Apochromat 100X/1.40 Oil DIC lens with 1.5X digital zoom.

2.13 | Scanning and transmission electron microscopy of ex vivo phagocytosis assays

Neutrophils (2.5×10^5 cells in 0.5 mL of supplemented RPMI medium) were enriched from amniotic fluid samples (Table 2) as described above, mixed with 50 μL of opsonized fluorescent-labeled bacteria in a 1.6 mL Eppendorf tube (Fisher Scientific), and incubated for 1 hour at 37°C. Next, the tube was centrifuged at 2300 *g* for 5 minutes and the supernatant was discarded. Electron microscopy fixative was carefully added to the cell pellet. Following fixation for 2 hours at 4°C, the cell pellet was gently washed with 1X electron microscopy wash buffer. As controls, pure bacteria were fixed and washed as described above. Cell pellets from amniotic fluid neutrophils plus bacteria or pure bacteria were transported to the Microscopy & Image Analysis Laboratory at the University of Michigan. Images were obtained using the AMRAY 1910 Field Emission Scanning Electron Microscope (SEMTechSolutions; North Billerica, MA, USA) and JEOL JSM-1400 plus transmission electron microscope.

3 | RESULTS

3.1 | Clinical characteristics of the study population

The first observation of in vivo phagocytosis by amniotic fluid neutrophils was made in 6 patients who were diagnosed with intra-amniotic infection (Table 1). All of the amniotic fluid samples had: (i) a positive microbiological culture, (ii) elevated concentrations of IL-6 (≥2.6 ng/mL[91]), (iii) increased WBC numbers (>50 cells/mm^3)[26] or viable cell counts (ie leukocytes; >100 cells/mm^3),[56] and (iv) a positive bacterial live/dead staining (Table 1). Most of the samples had low glucose concentrations (<14 mg/dL[113]) (Table 1). Four of these patients were diagnosed with clinical chorioamnionitis[10,15-18] (Table 1). The placentas from these patients presented lesions consistent with acute maternal and fetal inflammatory responses[106-108,117-121] (Table 1). The most common microorganisms found in these amniotic fluid samples were *U. urealyticum* and *M. hominis* followed by *S. agalactiae* (Table 1).

A total of 10 amniotic fluid samples from women with suspected intra-amniotic infection and/or inflammation were freshly collected for ex vivo phagocytosis assays (Table 2). All of the amniotic fluid samples had increased WBC counts (>50 cells/mm^3)[26] or viable cell counts (ie leukocytes; >100 cells/mm^3)[56] (Table 2). Seven of these patients were diagnosed with intra-amniotic infection as the amniotic fluid had a positive microbiological culture and elevated concentrations of IL-6 (≥2.6 ng/mL)[12,13,91-104] (Table 2). Six of these amniotic fluid samples had a positive bacterial live/dead staining (Table 2). The majority of the placentas from these patients presented lesions consistent with acute maternal and fetal inflammatory responses[106-108,117-121] (Table 2). The most common microorganisms found in the amniotic cavity were *U. urealyticum* and *M. hominis*; yet, Gram-positive and Gram-negative bacteria were also observed in women with intra-amniotic infection (Table 2).

3.2 | The first observation of phagocytosis by amniotic fluid neutrophils in women with intra-amniotic infection

While studying the morphological characteristics of amniotic fluid leukocytes using transmission electron microscopy, we observed that bacteria were engulfed by amniotic fluid neutrophils in cases with intra-amniotic infection. Sample 1 was from a patient who was diagnosed with intra-amniotic infection due to *S. agalactiae*, a Gram-positive coccus (Table 1). This bacterium seemed to be engulfed by an amniotic fluid neutrophil (Figure 1, sample 1, red arrows). Sample 2 was from a patient who was diagnosed with intra-amniotic infection caused by *Bacteroides fragilis* (Table 1), a Gram-negative bacillus. This rod-shaped bacterium seemed to have been ingested by an amniotic fluid neutrophil (Figure 1, sample 2, red arrow). Sample 3 was from a patient who was diagnosed with intra-amniotic infection due to *U. urealyticum* (Table 1). This bacterium lacks a cell wall; therefore, it was identified using bacterial live/dead staining, but not by Gram stain (Table 1). As mycoplasmas are similar to neutrophil intracellular organelles, we used transmission electron microscopy images of peripheral neutrophils without phagocytized bacteria to differentiate between cellular components and *U. urealyticum* (Fig. S1). Such a small bacterium was found engulfed in an amniotic fluid neutrophil (Figure 1, sample 3, red arrows). Sample 4 was from a patient who was diagnosed with polymicrobial intra-amniotic infection caused by *Enterobacter aerogenes*, *Enterococcus faecalis*, *M. hominis*, *Prevotella* spp., and *Streptococcus viridans* (Table 1). In this sample, a coccus was visualized inside of an amniotic fluid neutrophil (Figure 1, sample 4, red arrow). Sample 5 was from a second patient who was diagnosed with polymicrobial intra-amniotic infection caused by genital mycoplasmas (*U. urealyticum* and *M. hominis*), Gram-negative bacilli (*Prevotella* spp.), Gram-positive cocci (*S. agalactiae*), and Gram-positive bacilli (*Streptococcus anginosus*) (Table 1). In this sample, cocci were ingested by an amniotic fluid neutrophil (Figure 1, sample 5, red arrows). Sample 6 was from a third patient who was diagnosed with polymicrobial intra-amniotic infection caused by genital mycoplasmas (*U. urealyticum* and *M. hominis*) and *Prevotella* spp., a Gram-negative bacillus (Table 1). Yet, the Gram stain and bacterial live/dead staining revealed that this sample also had Gram-positive cocci, which were

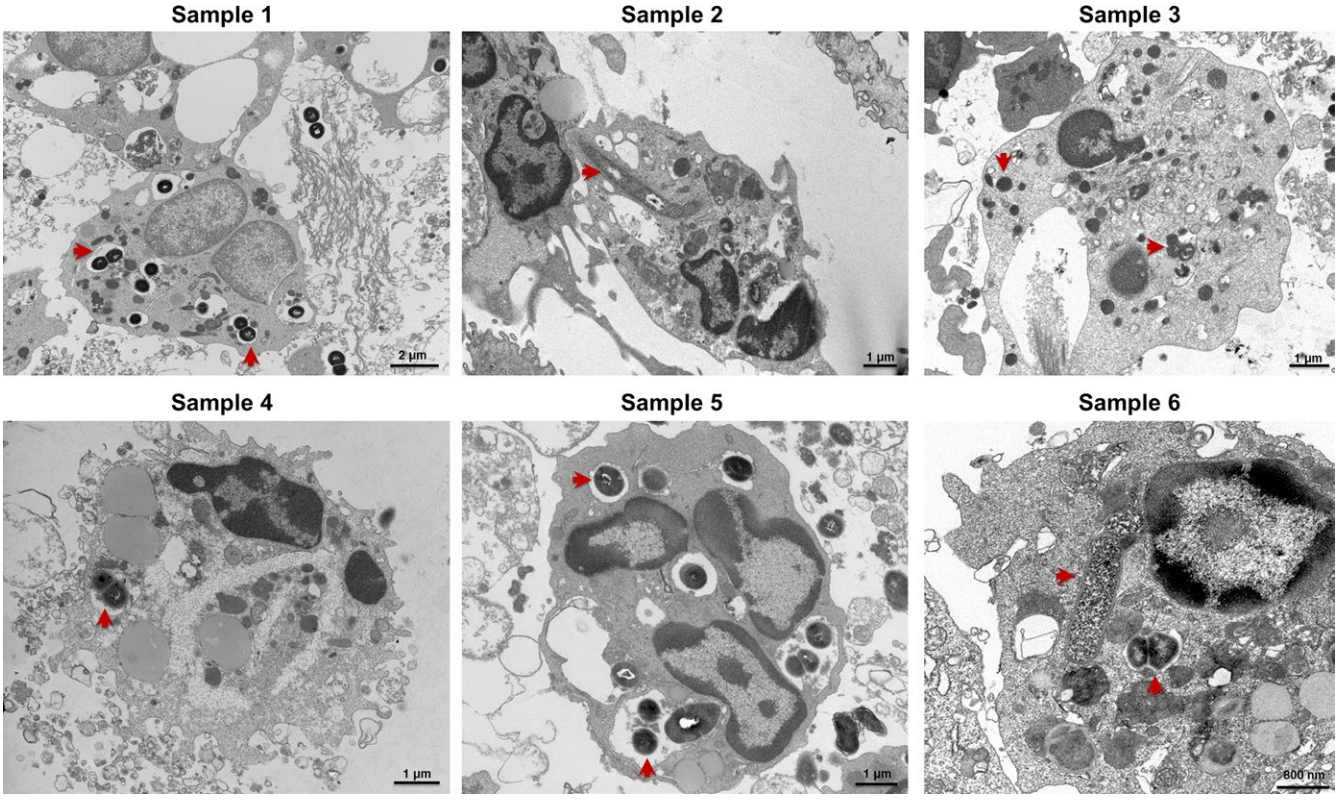

FIGURE 1 Amniotic fluid neutrophils from six women with intra-amniotic infection engulf bacteria in vivo. Transmission electron microscopy images from neutrophils observed in six amniotic fluid samples were captured at different magnifications (sample 1, 8000X; sample 2, 2000X; sample 3, 2500X; sample 4, 15 000X; sample 5, 2500X; and sample 6, 4000X). Red arrows identify bacteria ingested by amniotic fluid neutrophils

not identified using conventional microbiological cultivation methods (Table 1). We observed that amniotic fluid neutrophils ingested both bacillus- and coccus-shaped bacteria (Figure 1, sample 6, red arrows).

3.3 | Amniotic fluid neutrophils can rapidly phagocytize *Streptococcus agalactiae*

Most of the in vivo phagocytosis observations showed that amniotic fluid neutrophils can engulf cocci (Figure 1). The most common coccus found in the amniotic fluid of women with intra-amniotic infection is *S. agalactiae*.[13,122] Therefore, we first determined whether amniotic fluid neutrophils could phagocytize this bacterium. The morphology of *S. agalactiae* is shown by scanning electron microscopy in Figure 2A. When this bacterium was added to the amniotic fluid neutrophils, the cocci were rapidly phagocytized by these innate immune cells (Video S1). Prior to phagocytosis, *S. agalactiae* attached to the amniotic fluid neutrophils (Figure 2B, red arrow). Following phagocytosis, these cocci were engulfed by amniotic fluid neutrophils entirely and such a process was evidenced by confocal microscopy (Figure 2C, white arrows) and transmission electron microscopy (Figure 2D, red arrows). Semi-quantification of ex vivo phagocytosis assays revealed that amniotic fluid neutrophils phagocytized *S. agalactiae* as rapidly as peripheral neutrophils ($P>.05$; Figure 6).

3.4 | Amniotic fluid neutrophils can slowly phagocytize *Ureaplasma urealyticum*

Transmission electron microscopy revealed that amniotic fluid neutrophils can engulf *U. urealyticum* (Figure 1). This mycoplasma is the most common bacterium found in the amniotic cavity of women with intra-amniotic infection.[11,13,122] Using ex vivo phagocytosis assays, we next evaluated whether amniotic fluid neutrophils could phagocytize the bacterium. The morphology of *U. urealyticum* is shown by scanning electron microscopy in Figure 3A. The strain of *U. urealyticum* from ATCC was not phagocytized. When the strain of *U. urealyticum* isolated from a woman with intra-amniotic infection was added to the amniotic fluid neutrophils, the bacterium was slowly phagocytized by these innate immune cells (Video S2). Prior to phagocytosis, *U. urealyticum* attached to the amniotic fluid neutrophils (Figure 3B, red arrows). Following phagocytosis, this bacterium was observed engulfed in amniotic fluid neutrophils using confocal microscopy (Figure 3C, white arrow) and transmission electron microscopy (Figure 3D, red arrows). Semi-quantification of ex vivo phagocytosis assays revealed that amniotic fluid neutrophils phagocytized *U. urealyticum* at a slower speed compared to peripheral neutrophils ($P=.03$; Figure 6). Indeed, the ex vivo phagocytosis of *U. urealyticum* lasted longer than the phagocytosis of *S. agalactiae* ($P=.03$) and *G. vaginalis* ($P=.07$, 2.9 fold decrease) (Figure 6).

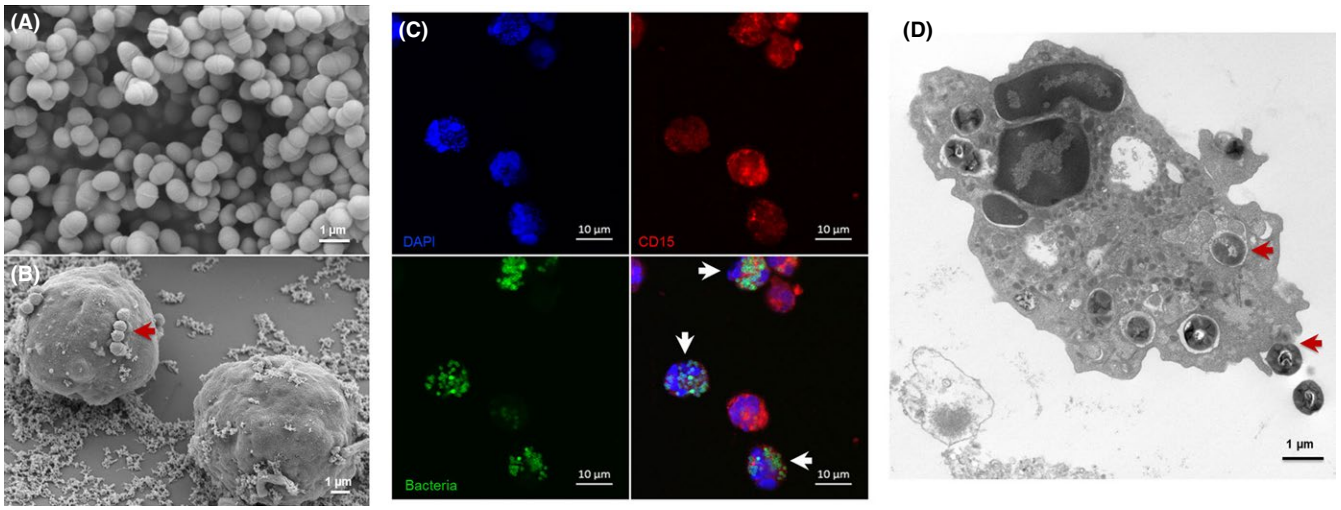

FIGURE 2 Amniotic fluid neutrophils can rapidly phagocytize *Streptococcus agalactiae*. (A) A scanning electron microscopy image of *S. agalactiae*. Magnification 10 000X. (B) A scanning electron microscopy image of amniotic fluid neutrophils and *S. agalactiae* (red arrow) prior to phagocytosis. Magnification 6000X. (C) Confocal microscopy images showing bacteria ingested by amniotic fluid neutrophils (white arrows). Separated images show DAPI staining in blue, CD15 (a neutrophil marker) in red, bacteria in green, and a merged image. Magnification 630X. (D) A transmission electron microscopy image of a neutrophil engulfing *S. agalactiae*. Magnification 2500X. Red arrows identify bacteria ingested by amniotic fluid neutrophils. N=5-9 each

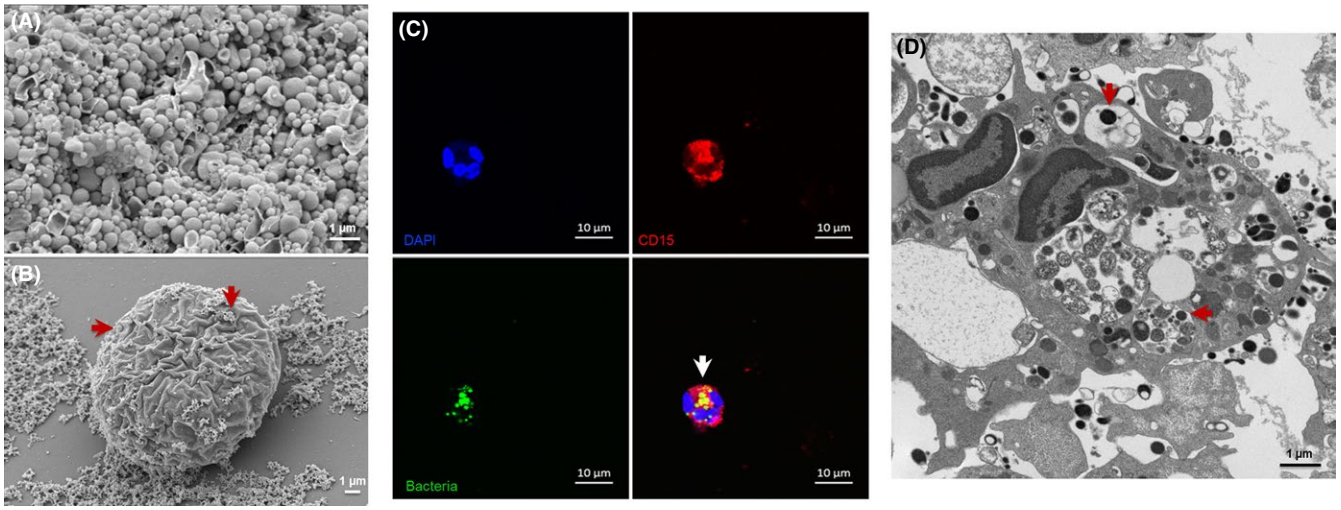

FIGURE 3 Amniotic fluid neutrophils can partially and slowly phagocytize *Ureaplasma urealyticum*. (A) A scanning electron microscopy image of *U. urealyticum*. Magnification 10 000X. (B) A scanning electron microscopy image of an amniotic fluid neutrophil and *U. urealyticum* (red arrows) prior to phagocytosis. Magnification 5000X. (C) Confocal microscopy images showing bacteria ingested by amniotic fluid neutrophils (white arrow). Separated images show DAPI staining in blue, CD15 (a neutrophil marker) in red, bacteria in green, and a merged image. Magnification 630X. (D) A transmission electron microscopy image of a neutrophil engulfing *U. urealyticum*. Magnification 2500X. Red arrows identify bacteria ingested by amniotic fluid neutrophils. N=5 each

3.5 | Amniotic fluid neutrophils can rapidly phagocytize *Gardnerella vaginalis*

Gardnerella vaginalis is frequently found in the amniotic cavity of women with polymicrobial infection.[13,14,122,123] Next, we determined whether amniotic fluid neutrophils could phagocytize a Gram-variable bacillus. The morphology of *G. vaginalis* is shown by scanning electron microscopy in Figure 4A. When this bacterium was added to the amniotic fluid neutrophils, the bacilli were rapidly phagocytized by these innate immune cells (Video S3). Prior to phagocytosis, *G. vaginalis* attached to the amniotic fluid neutrophils (Figure 4B, red arrow). Following phagocytosis, these bacilli were engulfed by amniotic fluid neutrophils and the process was revealed by confocal microscopy (Figure 4C, white arrows) and transmission electron microscopy (Figure 4D, red arrows). Semi-quantification of ex vivo phagocytosis assays revealed that amniotic fluid neutrophils phagocytized *G. vaginalis* as quickly as peripheral neutrophils ($P>.05$; Figure 6).

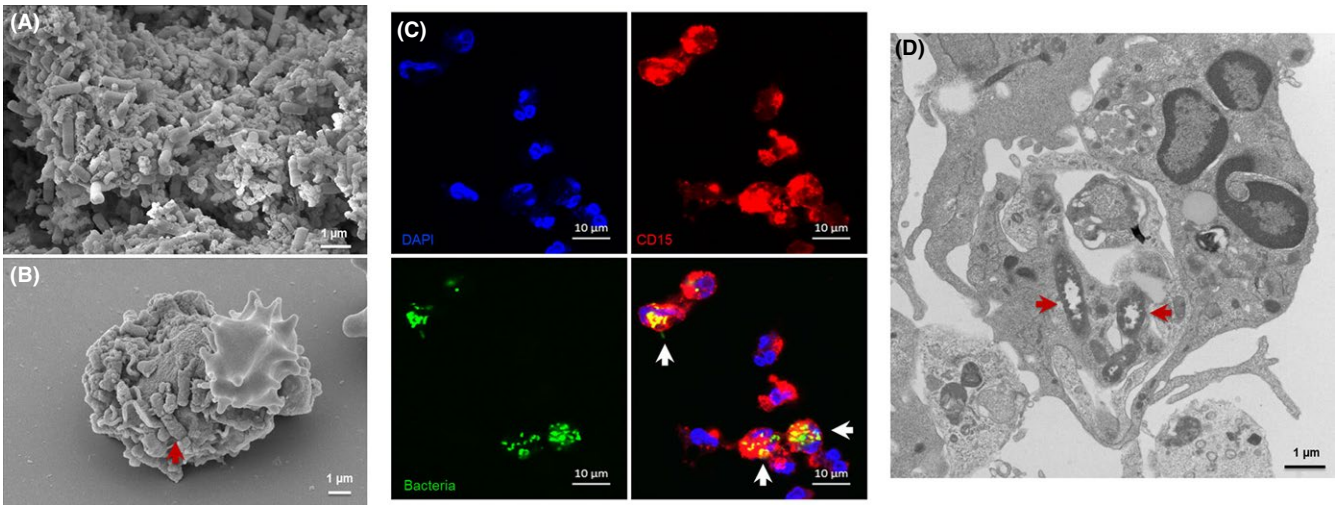

FIGURE 4 Amniotic fluid neutrophils can rapidly phagocytize *Gardnerella vaginalis*. (A) A scanning electron microscopy image of *G. vaginalis*. Magnification 5000X. (B) A scanning electron microscopy image of an amniotic fluid neutrophil and *G. vaginalis* (red arrow) prior to phagocytosis. Magnification 7500X. (C) Confocal microscopy images showing bacteria ingested by amniotic fluid neutrophils (white arrows). Separated images show DAPI staining in blue, CD15 (a neutrophil marker) in red, bacteria in green, and a merged image. Magnification 630X. (D) A transmission electron microscopy image of a neutrophil engulfing *G. vaginalis*. Magnification 3000X. Red arrows identify bacteria ingested by amniotic fluid neutrophils. N=5-8 each

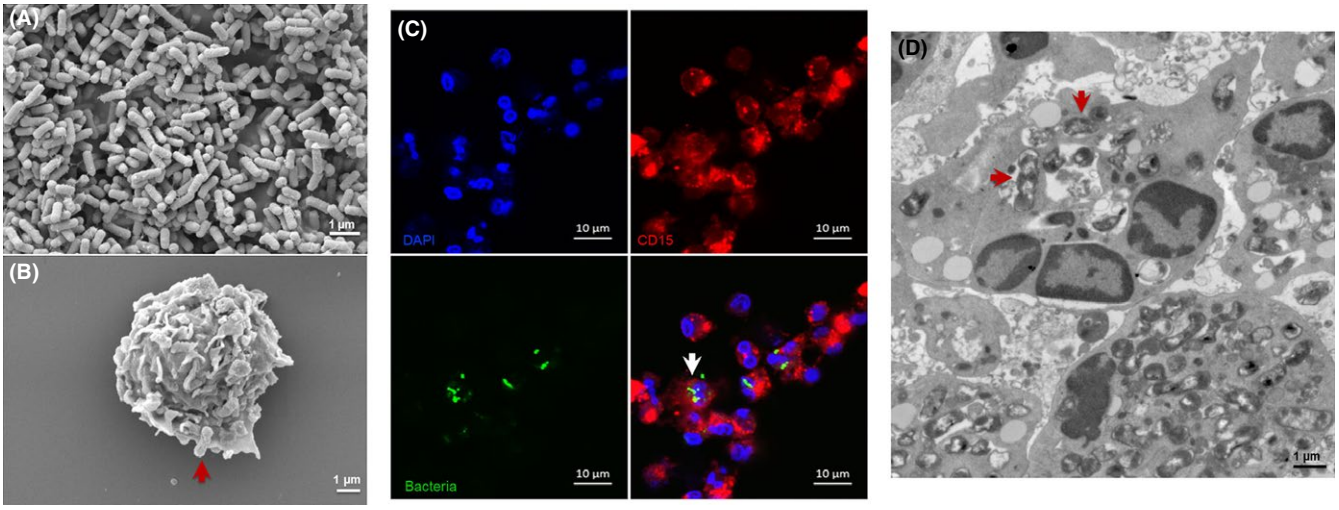

FIGURE 5 Amniotic fluid neutrophils can partially phagocytize *Escherichia coli*. (A) A scanning electron microscopy image of *E. coli*. Magnification 5000X. (B) A scanning electron microscopy image of an amniotic fluid neutrophil and *E. coli* (red arrow) prior to phagocytosis. Magnification 7500X. (C) Confocal microscopy images showing bacteria ingested by amniotic fluid neutrophils (white arrow). Separated images show DAPI staining in blue, CD15 (a neutrophil marker) in red, bacteria in green, and a merged image. Magnification 630X. (D) A transmission electron microscopy image of a neutrophil engulfing *E. coli*. Magnification 1200X. Red arrows identify bacteria ingested by amniotic fluid neutrophils. N=5-9 each

3.6 | Amniotic fluid neutrophils can partially phagocytize *Escherichia coli*

Escherichia coli is a Gram-negative bacillus, which has also been observed in the amniotic cavity of women with intra-amniotic infection.[13,14,122] Therefore, we determined whether amniotic fluid neutrophils could phagocytize the rod-shaped bacterium. The morphology of *E. coli* is shown by scanning electron microscopy in Figure 5A. When this bacterium was added to the amniotic fluid neutrophils, the bacilli were partially phagocytized by these innate immune cells (Video S4). Prior to phagocytosis, *E. coli* attached to the amniotic fluid neutrophils (Figure 5B, red arrow). Following phagocytosis, a few bacilli were engulfed by amniotic fluid neutrophils and the process was evidenced by confocal microscopy (Figure 5C, white arrow) and transmission electron microscopy (Figure 5D, red arrows). Semi-quantification of ex vivo phagocytosis assays revealed that amniotic fluid neutrophils phagocytized *E. coli* slower than peripheral neutrophils (P=.003; Figure 6). Yet, phagocytosis of *E. coli* by amniotic fluid neutrophils was not as delayed as in the case of *U. urealyticum* (Figure 6).

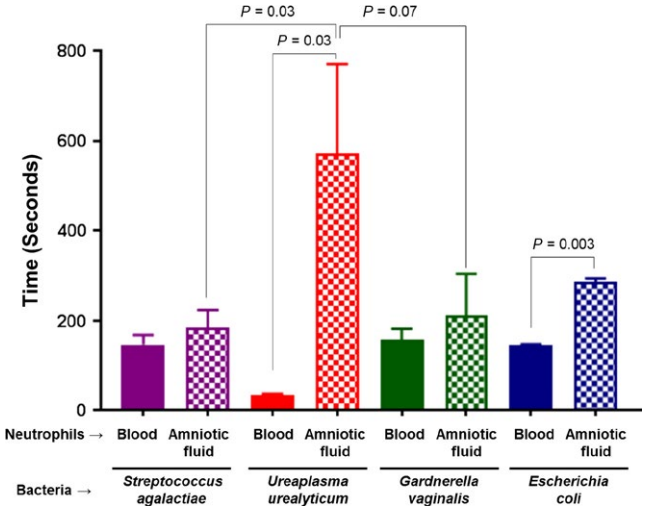

FIGURE 6 Semi-quantification of ex vivo phagocytosis assays. Confocal time series of peripheral and amniotic fluid neutrophils phagocytizing *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia coli* were recorded at 7.75-s time intervals from 5 to 20 min. A semi-quantification of the time-interval for each ex vivo phagocytosis assay was calculated based on the duration of the assay and the number of frames taken per experiment

4 | DISCUSSION

Neutrophils are the most abundant leukocytes found in the amniotic cavity of women with intra-amniotic infection and/or inflammation.[26,31] Amniotic fluid neutrophils participate in the innate immune host defense mechanisms that take place in the amniotic cavity of women with intra-amniotic infection.[48-50] As a result, these innate immune cells release antimicrobial peptides[53,55] and cytokines/chemokines[31] as well as trap and kill bacteria by forming NETs.[56] In the study herein, we provide in vivo and ex vivo evidence that amniotic fluid neutrophils can phagocytize bacteria associated with intra-amniotic infection; yet, differences in the rapidity of phagocytosis were observed among the studied microorganisms.

Amniotic fluid neutrophils can rapidly phagocytize *Streptococcus* spp., including *S. agalactiae*, which is commonly found in the amniotic fluid of women with intra-amniotic infection.[13,122] The process of phagocytosis for *S. agalactiae* is mediated by toll-like receptors (eg TLR2) and integrins (eg CD11b/CD18).[124] However, this coccus can also evade neutrophil phagocytosis by binding to sialic acid-binding immunoglobulin-like lectin 5, a protein expressed on the surface of phagocytes.[125] Interestingly, in cases with polymicrobial intra-amniotic infection, cocci were the most commonly observed microorganisms engulfed by amniotic fluid neutrophils, suggesting that these innate immune cells prefer to engulf this genus. This finding is consistent with a previous report demonstrating that neutrophils favor the ingestion of Gram-positive cocci over Gram-negative bacilli.[126] Yet, our ex vivo phagocytosis assays showed that amniotic fluid neutrophils can also phagocytize bacilli. Another important observation is that the phagocytosis of *S. agalactiae* by amniotic fluid neutrophils was quicker than for the other bacteria. This finding indicates that when GBS invades the amniotic cavity, amniotic fluid neutrophils can rapidly kill these bacteria as a mechanism of host defense.

Ureaplasma urealyticum is the most common bacterium present in the amniotic cavity of women with intra-amniotic infection.[11,13,122] Herein, we found that amniotic fluid neutrophils can phagocytize this genital mycoplasma. However, this process was slower than with other bacteria. In addition, in cases with polymicrobial intra-amniotic infection, we could not find *U. urealyticum* engulfed in amniotic fluid neutrophils, suggesting that this bacterium was not always phagocytized. These results are consistent with previous reports demonstrating that *U. urealyticum*, as well as *M. hominis*, can circumvent phagocytosis and even survive if ingested.[127-130] Mycoplasmas can evade phagocytosis by: (i) producing proteases, lipases, phospholipases, and oxygen radicals, which can block the creation or maturation of the phagosome,[131-133] (ii) producing ammonia which can impair the phagosome-lysosome fusion,[134,135] or (iii) internalizing into the cytoplasm of phagocytes (mechanism unknown).[130,136] In fact, it was suggested that neutrophils do not participate in the host defense mechanisms against mycoplasmas and may even aid in the dissemination of the infection.[129] Taken together, these data suggest that amniotic fluid neutrophils cannot efficiently kill *U. urealyticum*, and this might explain why most intra-amniotic infections are associated with these microorganisms. Nevertheless, further research is needed to evaluate the efficiency of amniotic fluid neutrophils to phagocytize genital mycoplasmas, and whether such bacteria can evade and survive this mechanism of microbial killing in the amniotic cavity.

Gardnerella vaginalis is found in the amniotic cavity of women with polymicrobial infection[13,14,122,123] and can induce a strong pro-inflammatory response in the chorioamniotic membranes.[137] Amniotic fluid neutrophils could rapidly phagocytize this Gram-variable bacillus, a process likely mediated by the activation of the alternative pathway of the complement system.[138] The current study also provides evidence that amniotic fluid neutrophils can phagocytize Gram-negative bacillus, including *B. fragilis*, *Prevotella* spp., and *E. coli*. However, the phagocytosis of *E. coli* by amniotic fluid neutrophils was not as efficient as in cases with *S. agalactiae*. A possible explanation for this impairment is that *E. coli* uses its capsular antigens O75 and K5 to resist neutrophil phagocytosis.[139] In the event that *E. coli* is phagocytized, this bacillus is able to survive the bactericidal activity of the neutrophils and live within these innate immune cells.[140] Together, these data allow us to propose that amniotic fluid neutrophils can engulf bacilli; yet, their phagocytic efficiency may be different among genera.

A central question that requires further investigation is whether amniotic fluid neutrophils in cases with intra-amniotic infection and/or inflammation are of maternal and/or fetal origin. These innate immune cells are thought to be predominantly of fetal origin[141,142] and invade the amniotic cavity by migrating from the fetal vessels of the chorionic plate.[143] However, abundant neutrophils have also been observed in the amniotic fluid of patients with a severe maternal inflammatory response but without a fetal inflammatory response, indicating that there is a possibility that these innate immune cells are of maternal origin or a mixture of both fetal and maternal neutrophils.[31,56] This

question is relevant as cord blood neutrophils display differences in functionality compared to peripheral neutrophils.[144-148] Indeed, cord blood neutrophils can phagocytize *E. coli* and *Streptococcus pyogenes* but not *S. agalactiae*,[149] suggesting that the phagocytosis of GBS observed in our study was performed by amniotic fluid neutrophils of maternal origin or that the amniotic fluid components enhance the phagocytic ability of fetal neutrophils. Moreover, cord blood neutrophils from preterm neonates exhibit impaired innate immune responses, including phagocytosis, compared to term neonates.[150-152] Therefore, it is essential to investigate the origin of amniotic fluid neutrophils in cases with intra-amniotic infection.

In summary, we report that amniotic fluid neutrophils can phagocytize bacteria found in the lower genital track, namely *S. agalactiae*, *U. urealyticum*, *G. vaginalis*, and *E. coli*. However, amniotic fluid neutrophils seem to display a delayed ability to phagocytize *U. urealyticum* and *E. coli*. These findings provide a host defense mechanism whereby amniotic fluid neutrophils can kill microbes invading the amniotic cavity.

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CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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