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Amniotic Fluid Neutrophils Can Phagocytize Bacteria: A Mechanism for Microbial Killing in the Amniotic Cavity

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Abstract

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—1) Intra-amniotic infection-related bacteria including cocci (e.g. *Streptococcus agalactiae*), bacilli (e.g. *Bacteriodes fragilis* and *Prevotella spp.*), and small bacteria without a cell wall (e.g. *Ureaplasma urealyticum*) were found inside of amniotic fluid neutrophils; 2) peripheral neutrophils (controls) rapidly phagocytized *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia coli*; 3) amniotic fluid neutrophils can rapidly phagocytize *Streptococcus agalactiae* and *Gardnerella vaginalis*; and 4) amniotic fluid neutrophils can slowly

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phagocytize *Ureaplasma urealyticum* and *Escherichia coli*; yet, the process of phagocytosis of the genital mycoplasma was lengthier.

Conclusions—Amniotic fluid neutrophils can phagocytize bacteria found in the amniotic cavity of women with intra-amniotic infection, namely *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia 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.

Graphical abstract

Keywords

Acute chorioamnionitis; Clinical chorioamnionitis; Cytokines; 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

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 others¹⁰⁻¹⁴. This inflammatory response can result in systemic^{10, 15-18} and/or local¹⁹⁻²⁵ inflammatory responses. Systemically, intra-amniotic infection can be manifested as clinical chorioamnionitis, which refers to the presence of maternal fever associated with clinical signs (i.e. foul-smelling discharge, 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 count²⁶⁻³¹ and elevated concentrations of cytokines^{8, 32} and lipid mediators (e.g. prostaglandins)³³⁻⁴⁷ in the amniotic cavity.

The most abundant white blood cells (i.e. leukocytes) in the amniotic cavity of women with intra-amniotic infection are the neutrophils, and 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 infection⁴⁸⁻⁵⁰. Indeed, amniotic fluid neutrophils are a source of anti-microbial products⁵¹⁻⁵⁵ and cytokines³¹. In addition, these innate immune cells can trap and

kill bacteria invading the amniotic cavity by forming neutrophil extracellular traps or NETs⁵⁶. Neutrophils infiltrating the chorioamniotic membranes also form NETs in cases with acute histologic chorioamnionitis⁵⁷, a placental lesion associated with elevated concentrations of pro-inflammatory cytokines in the amniotic fluid^{32, 48, 58-75}. The formation of NETs or NETosis⁷⁶ represents the final containment effort of a neutrophil to kill pathogens⁷⁷. NETs are web-like structures composed of DNA, histones, and anti-microbial products that trap and/or eliminate microbes through their biochemical components⁷⁷⁻⁸⁰. Yet, only a fraction (~20%) of human neutrophils⁸¹, including those in the amniotic cavity⁵⁶, 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 phagocytosis⁸²⁻⁸⁴, a main mechanism for microbial killing⁸⁵. Phagocytosis is the receptor-mediated process whereby a cell (e.g. neutrophil) extends its plasma membrane around the target (e.g. 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 complex⁸⁶. 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 their phagosome are different to those made by other phagocytes (e.g. macrophages)⁸⁷. The antimicrobial effect of the neutrophil phagosome is due to the fusion of their granules with secretory vesicles, which contain albumin and express alkaline phosphatase and CD35 on their membranes⁸⁶. Neutrophils contain three types of cytoplasmic granules: 1) primary (or azurophilic) granules, which are positive for peroxidase and have lytic enzymes and defensins; 2) secondary granules (or specific granules), which contain lactoferrin; and 3) 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 in order to kill microbes without causing tissue damage to the host.

Since neutrophil phagocytosis is a main host defense mechanism for microbial killing, we investigated whether: 1) intra-amniotic infection-related bacteria were found engulfed in amniotic fluid neutrophils using transmission electron microscopy; 2) amniotic fluid neutrophils could phagocytize bacteria associated with intra-amniotic infection (*Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia 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.

Materials and Methods

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 – 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) in order to obtain the viable cell numbers. Most of the viable cells are leukocytes³¹. The inclusion criteria were (1) singleton gestations, (2) samples without blood contamination, and (3) sufficient amniotic fluid leukocytes ($>1 \times 10^6$ cells/mL) to evaluate *in vivo* phagocytosis using transmission electron microscopy or perform *ex vivo* phagocytosis assays coupled with live imaging, confocal microscopy as well as 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, U. S. Department of Health and Human Services (NICHD/NIH/DHHS).

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 $>37.8^{\circ}\text{C}$) accompanied by two or more of the following criteria: 1) uterine tenderness; 2) malodorous vaginal discharge; 3) fetal tachycardia (heart rate >160 beats/min); 4) maternal tachycardia (heart rate >100 beats/min); and 5) maternal leukocytosis (leukocyte count $>15,000$ cells/mm³). 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, 92}. Intra-amniotic infection was defined as the presence of MIAC with intra-amniotic inflammation^{12, 13, 91-104}.

Placental histopathological examinations

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 disc 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 Redline's

criteria¹⁰⁵⁻¹⁰⁸. While the stage of the placenta lesion refers to the progression of the inflammatory process based on the anatomical regions infiltrated by neutrophils (stage 1-3), the grade of the placenta lesion is defined by the intensity of the acute inflammatory process at a particular site [grade 1 (mild to moderate) and grade 2 (severe)]^{105, 107}. For more information about the staging and grading of the acute inflammatory lesions of the placenta, please see placental pathology reviews by Kim et al.¹⁰⁷ and Redline RW¹⁰⁸.

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, white blood cell (WBC) count in amniotic fluid samples was determined by using a hemocytometer chamber, according to methods previously described²⁶. Glucose concentration was also determined¹¹³ and Gram stain¹¹⁴ 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, since 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.

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.

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, New York) in a sterile biosafety cabinet. Briefly, 100µL of amniotic fluid were mixed with 900µL of sterile 1× phosphate buffered saline (PBS; Life Technologies). Three microliters of the dye mix (Component A and B were mixed at a 1:1 ratio) were added to the cell suspension and incubated for 15 min at room temperature in the dark. Next, the cells were centrifuged at 10,000 × g for 5 min and the supernatant was discarded. The cell pellet was then re-suspended in 5µL of 1× PBS, and a slide smear was prepared and air-dried. Lastly, the slide was gently rinsed with 1× 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).

Transmission electron microscopy of *in vivo* phagocytosis

Amniotic fluid samples (Table 1) were passed through a sterile 15µm filter (Cat# 43-50015-03, pluriSelect Life Science; Leipzig, Germany), centrifuged at $2300 \times g$ for 5 min at room temperature and the supernatant was discarded. Electron microscopy fixative (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4; Cat# 16537-05, Electron Microscopy Science, Hatfield, PA) was carefully added to the cell pellet. Following fixation for 2 hours at 4°C, the cell pellet was gently washed with $1 \times$ electron microscopy wash buffer (Sorensen's phosphate buffer 0.2 M, 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/microscopy-image-analysis>). Images were obtained using a JEOL JSM-1400 plus transmission electron microscope (JEOL, Peabody, MA).

Bacteria strains and growth conditions for *ex vivo* phagocytosis assays

Streptococcus agalactiae (ATCC® 13813), *Ureaplasma urealyticum* (ATCC® 27618), *Gardnerella vaginalis* (ATCC® 14018) and *Escherichia coli* (*E. coli*, ATCC® 700926) were purchased from American Type Culture Collection (ATCC, Manassas, VA). *Ureaplasma urealyticum* was also isolated from a patient with intra-amniotic infection. *Streptococcus agalactiae* and *Gardnerella vaginalis* were cultured in brain heart infusion broth (BHI, Cat#R060260, Remel, Lenexa, KS) at 37 °C with shaking at 180 rpm. *Escherichia coli* was grown in Luria-Bertani broth (LB, Cat#L7658, Sigma, Saint Louis, MO) 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 $2,300 \times g$ for 5 min and re-suspended in $1 \times$ PBS. *Ureaplasma urealyticum* obtained from ATCC or a clinical sample was cultured in SP4 Broth with urea (Hardy Diagnostics, Santa Maria, CA) at 37°C with shaking at 180 rpm until a color change (yellow to pink) was observed. The culture broth was then centrifuged at $1,500 \times g$ for 30 min at 4°C. The identification of characteristic colonies of *Ureaplasma urealyticum* was performed on an A8 agar plate (Hardy Diagnostics).

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 $1 \times$ PBS and sodium bicarbonate solution was added to a final concentration of 0.1M. 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 $1 \times$ PBS containing 20% glycerol (Cat#G1796, TEKnova, Hollister, CA) to an OD_{600} of 0.3 ($\sim 1.5 \times 10^7/50\mu\text{L}$). Fluorescent-labeled bacteria were aliquoted and stored in -80°C until use.

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) for 30 min at 37°C with a gentle rotation. Bacteria were washed with $1 \times$ 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 as well as scanning and transmission electron microscopy.

Live imaging of *ex vivo* phagocytosis assays

Amniotic fluid samples (Table 2) were passed through a sterile 15µm filter and centrifuged at $200 \times g$ for 5 min at room temperature. This step allows the enrichment of amniotic fluid leukocytes (mostly neutrophils³¹) and the elimination of epithelial cells⁵⁶. Amniotic fluid leukocytes were then re-suspended in supplemented RPMI medium at a concentration of 2.5×10^5 cells/0.5mL, plated in a 35mm culture dish with a cover glass bottom (MatTek, Ashland, MA), and labeled with an anti-human CD15-PE-CF594 antibody (Clone W6D3, Cat#562372, BD Biosciences, San Jose, CA). 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 63×/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 50µL 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 mins. 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; St. Louis, Missouri), according to the manufacturer's instructions and a previously published method¹¹⁶. Briefly, 6 mL of peripheral blood were layered on top of 6 mL of Histopaque 1119 and centrifuged at $800 \times g$ 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 $1 \times$ PBS (Life Technologies). Purified neutrophils were then incubated with labeled bacteria for *ex vivo* phagocytosis assays and a semi-quantification was performed as described above.

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) containing 12-mm cover slips (Fisher Scientific, Waltham, MA) at a concentration of 2.5×10^5 cells/0.5mL for 1 hour at 37°C in supplemented RPMI medium. Following the attachment of neutrophils to the cover slips, medium was replaced with 200µL of fresh medium and 20µL of an anti-human CD15-PE-CF594 antibody was added to the culture dish. After 30 min of incubation, excess

antibody was removed by gently washing the amniotic fluid neutrophils with supplemented RPMI medium. Next, 500 μ L of fresh medium and 50 μ L of opsonized fluorescent-labeled bacteria were added to the amniotic fluid neutrophils. The culture plate was then centrifuged at 600 $\times$ 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, Delaware) 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 100 $\times$ /1.40 Oil DIC lens with 1.5 $\times$ digital zoom.

Scanning and transmission electron microscopy of *ex vivo* phagocytosis assays

Neutrophils (2.5 $\times$ 10⁵ cells in 0.5mL of supplemented RPMI medium) were enriched from amniotic fluid samples (Table 2) as described above, mixed with 50 μ L 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 2,300 $\times$ g for 5 min 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 1 $\times$ 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 an AMRAY 1910 Field Emission Scanning Electron Microscope (SEMTechSolutions; North Billerica, MA) and JEOL JSM-1400 plus transmission electron microscope.

Results

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: 1) a positive microbiological culture, 2) elevated concentrations of IL-6 (2.6 ng/mL^{91, 92}), 3) increased white blood cell numbers (>50 cells/mm³)²⁶ or viable cell counts (i.e. leukocytes; >100 cells/mm³)⁵⁶, and 4) a positive bacterial live/dead staining (Table 1). Most of the samples had low glucose concentrations (<14 mg/dL¹¹³) (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 *Ureaplasma urealyticum* and *Mycoplasma hominis* followed by *Streptococcus 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 white blood cell counts (>50 cells/mm³)²⁶ or viable cell counts (i.e. leukocytes; >100 cells/mm³)⁵⁶ (Table 2). Seven of these patients

were diagnosed with intra-amniotic infection since 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 *Ureaplasma urealyticum* and *Mycoplasma hominis*; yet, Gram positive and Gram negative bacteria were also observed in women with intra-amniotic infection (Table 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 *Streptococcus agalactiae*, which is a Gram positive coccus (Table 1). Such 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), which is a Gram negative bacillus. Such 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 *Ureaplasma 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). Since 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 *Ureaplasma urealyticum* (Supplementary Figure 1). 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*, *Mycoplasma 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 (*Ureaplasma urealyticum* and *Mycoplasma hominis*), Gram negative bacilli (*Prevotella spp.*), and Gram positive (*Streptococcus agalactiae*) and negative (*Streptococcus anginosus*) bacilli (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 (*Ureaplasma urealyticum* and *Mycoplasma hominis*) and *Prevotella spp.*, which is 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 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).

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 *Streptococcus agalactiae*^{13, 122}. Therefore, we first determined whether amniotic fluid neutrophils could phagocytize such a bacterium. The morphology of *Streptococcus agalactiae* is shown by scanning electron microscopy in Figure 2A. When this bacterium was added to the amniotic fluid neutrophils, such cocci were rapidly phagocytized by these innate immune cells (Video 1). Prior to phagocytosis, *Streptococcus 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 *Streptococcus agalactiae* as rapidly as peripheral neutrophils ($p>0.05$; Figure 6).

Amniotic fluid neutrophils can slowly phagocytize *Ureaplasma urealyticum*

Transmission electron microscopy revealed that amniotic fluid neutrophils can engulf *Ureaplasma urealyticum* (Figure 1). Such a 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 such a bacterium. The morphology of *Ureaplasma urealyticum* is shown by scanning electron microscopy in Figure 3A. The strain of *Ureaplasma urealyticum* from ATCC was not phagocytized. When the strain of *Ureaplasma urealyticum* isolated from a woman with intra-amniotic infection was added to the amniotic fluid neutrophils, such a bacterium was slowly phagocytized by these innate immune cells (Video 2). Prior to phagocytosis, *Ureaplasma 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 *Ureaplasma urealyticum* at a slower speed compared to peripheral neutrophils ($p=0.03$; Figure 6). Indeed, the *ex vivo* phagocytosis of *Ureaplasma urealyticum* lasted longer than the phagocytosis of *Streptococcus agalactiae* ($p=0.03$) and *Gardnerella vaginalis* ($p=0.07$, 2.9 fold decrease) (Figure 6).

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 such a Gram variable bacillus. The morphology of *Gardnerella vaginalis* is shown by scanning electron microscopy in Figure 4A. When this bacterium was added to the amniotic fluid neutrophils, such bacilli were rapidly phagocytized by these innate immune cells (Video 3). Prior to phagocytosis, *Gardnerella vaginalis* attached to the amniotic fluid neutrophils (Figure 4B, red arrow). Following phagocytosis, these bacilli were engulfed by amniotic fluid neutrophils and such a 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 *Gardnerella vaginalis* as quickly as peripheral neutrophils ($p>0.05$; Figure 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 such a rod-shaped bacterium. The morphology of *Escherichia coli* is shown by scanning electron microscopy in Figure 5A. When this bacterium was added to the amniotic fluid neutrophils, such bacilli were quickly phagocytized by these innate immune cells (Video 4). Prior to phagocytosis, *Escherichia coli* attached to the amniotic fluid neutrophils (Figure 5B, red arrow). Following phagocytosis, a few bacilli were engulfed by amniotic fluid neutrophils and such a 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 *Escherichia coli* slower than peripheral neutrophils ($p=0.003$; Figure 6). Yet, phagocytosis of *Escherichia coli* by amniotic fluid neutrophils was not as delayed as in the case of *Ureaplasma urealyticum* (Figure 6).

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 infection⁴⁸⁻⁵⁰. As a result, these innate immune cells release antimicrobial peptides^{53, 55} and cytokines/chemokines³¹ as well as trap and kill bacteria by forming NETs⁵⁶. 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 was observed among the studied microorganisms.

Amniotic fluid neutrophils can rapidly phagocytize *Streptococcus spp*, including *Streptococcus agalactiae*, which is commonly found in the amniotic fluid of women with intra-amniotic infection^{13, 122}. The process of phagocytosis for *Streptococcus agalactiae* is mediated by toll-like receptors (e.g. TLR2) and integrins (e.g CD11b/CD18)¹²⁴. Yet, this coccus can also evade neutrophil phagocytosis by binding to sialic acid-binding immunoglobulin-like lectin 5, a protein expressed on the surface of phagocytes¹²⁵. 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 bacillus¹²⁶. Yet, our *ex vivo* phagocytosis assays showed that amniotic fluid neutrophils can also phagocytize bacilli. Another important observation is that the phagocytosis of *Streptococcus 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 found in the amniotic cavity of women with intra-amniotic infection^{11, 13, 122}. Herein, we found that amniotic fluid neutrophils can phagocytize such a genital mycoplasma. However, this process was slower than with other bacteria. In addition, in cases with polymicrobial intra-amniotic infection, we could not find *Ureaplasma urealyticum* engulfed in amniotic fluid neutrophils, suggesting that this bacterium was not always phagocytized. These results are consistent with previous reports demonstrating that *Ureaplasma urealyticum*, as well as *Mycoplasma hominis*, can circumvent phagocytosis and even survive if ingested¹²⁷⁻¹³⁰. Mycoplasmas can evade phagocytosis by: 1) producing proteases, lipases, phospholipases, and oxygen radicals, which can block the creation or maturation of the phagosome¹³¹⁻¹³³, 2) producing ammonia which can impair the phagosome-lysosome fusion^{134, 135}, or 3) 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 infection¹²⁹. Taken together, these data suggest that amniotic fluid neutrophils cannot efficiently kill *Ureaplasma urealyticum*, and this might explain why most intra-amniotic infections are due to these microorganisms. Nevertheless, further research is needed in order 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 membranes¹³⁷. Amniotic fluid neutrophils could rapidly phagocytize this Gram variable bacillus, a process that is likely mediated by the activation of the alternative pathway of the complement system¹³⁸. The current study also provides evidence that amniotic fluid neutrophils can phagocytize Gram negative bacillus, including *Bacteroides fragilis*, *Prevotella spp.*, and *Escherichia coli*. However, the phagocytosis of *Escherichia coli* by amniotic fluid neutrophils was not as efficient as in cases with *Streptococcus agalactiae*. A possible explanation for this impairment is that *Escherichia coli* uses its capsular antigens O75 and K5 to resist neutrophil phagocytosis¹³⁹. In the event that *Escherichia coli* is phagocytized, this bacillus is able to survive the bactericidal activity of the neutrophils and live within these innate immune cells¹⁴⁰. 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 plate¹⁴³. However, abundant neutrophils have also been observed in the amniotic fluid of patients with a severe maternal inflammatory response 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 since cord blood neutrophils

display differences in functionality compared to peripheral neutrophils¹⁴⁴⁻¹⁴⁸. Indeed, cord blood neutrophils can phagocytize *Escherichia coli* and *Streptococcus pyogenes* but not *Streptococcus 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 neonates¹⁵⁰⁻¹⁵². 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 *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia coli*. However, amniotic fluid neutrophils seem to display a delayed ability to phagocytize *Ureaplasma urealyticum* and *Escherichia coli*. These findings provide a host defense mechanism whereby amniotic fluid neutrophils can kill microbes invading the amniotic cavity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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, 8,000 $\times$; sample 2, 2,000 $\times$; sample 3, 2,500 $\times$; sample 4, 15,000 $\times$; sample 5, 2,500 $\times$; and sample 6, 4,000 $\times$). Red arrows identify ingested bacteria by amniotic fluid neutrophils.

Figure 2.

Amniotic fluid neutrophils can rapidly phagocytize *Streptococcus agalactiae*. A) A scanning electron microscopy image of *Streptococcus agalactiae*. Magnification 10,000 $\times$. B) A scanning electron microscopy image of amniotic fluid neutrophils and *Streptococcus agalactiae* (red arrow) prior to phagocytosis. Magnification 6,000 $\times$. C) Confocal microscopy images showing ingested bacteria 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 630 $\times$. D) A transmission electron microscopy image of a neutrophil engulfing *Streptococcus agalactiae*. Magnification 2,500 $\times$. Red arrows identify ingested bacteria 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 *Ureaplasma urealyticum*. Magnification 10,000 $\times$. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Ureaplasma urealyticum* (red arrows) prior to phagocytosis. Magnification 5,000 $\times$. C) Confocal microscopy images showing ingested bacteria 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 630 $\times$. D) A transmission electron microscopy image of a neutrophil engulfing *Ureaplasma urealyticum*. Magnification 2,500 $\times$. Red arrows identify ingested bacteria by amniotic fluid neutrophils. N=5 each.

Figure 4.

Amniotic fluid neutrophils can rapidly phagocytize *Gardnerella vaginalis*. A) A scanning electron microscopy image of *Gardnerella vaginalis*. Magnification 5,000 $\times$. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Gardnerella vaginalis* (red arrow) prior to phagocytosis. Magnification 7,500 $\times$. C) Confocal microscopy images showing ingested bacteria 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 630 $\times$. D) A transmission electron microscopy image of a neutrophil engulfing *Gardnerella vaginalis*. Magnification 3,000 $\times$. Red arrows identify ingested bacteria 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 *Escherichia coli*. Magnification 5,000 $\times$. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Escherichia coli* (red arrow) prior to phagocytosis. Magnification 7,500 $\times$. C) Confocal microscopy images showing ingested bacteria 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 630 $\times$. D) A transmission electron microscopy image of a neutrophil engulfing *Escherichia coli*. Magnification 1,200 $\times$. Red arrows identify ingested bacteria by amniotic fluid neutrophils. N=5-9 each.

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-second time intervals from 5 to 20 mins. 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.

Table 1

Clinical characteristics of amniotic fluid samples in which *in vivo* phagocytosis was observed.

Sample	Clinical Chorioamnionitis	Viable Cell Count* (cells/mm ³)	Gestational Age at Amniocentesis	Collection Method of 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	
1	Yes	2200	36.6	Transabdominal	8.1	Gram Positive Cocci	Positive	<i>Streptococcus agalactiae</i>	310	<1	36.7	Acute Maternal inflammatory Response	Acute Fetal Inflammatory Response
2	No	100	18.9	Transabdominal	121.3	Gram Negative Bacilli	Positive	<i>Bacteroides fragilis</i>	65	20	19.6	Stage 3	Stage 2
3	Yes	18800	40	C/S	47.6	Negative	Positive	<i>Ureaplasma urealyticum</i>	NA	NA	40	Stage 2	Stage 2
4	No	9920	23	Transabdominal	27	Gram Positive cocci, Few Gram negative coccobacilli	Positive	<i>Enterobacter aerogenes</i> , <i>Enterococcus faecalis</i> , <i>Mycoplasma hominis</i> , <i>Prevotella</i> spp, <i>Streptococcus viridans</i>	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	<i>Ureaplasma urealyticum</i> , <i>Mycoplasma hominis</i> , <i>Streptococcus agalactiae</i> , <i>Streptococcus anginosus</i> , <i>Prevotella</i> spp	4000	<1	35.6	Stage 2	Stage 3
6	Yes	6780	35.6	C/S	NA	Gram Positive Cocci, Gram Negative Bacilli	Positive	<i>Ureaplasma urealyticum</i> , <i>Mycoplasma hominis</i> , <i>Prevotella</i> spp	7920	<1	35.6	Stage 2	Stage 3

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

* 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* (cells/mm ³)	Gestational Age at Amniocentesis	Collection Method of 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	<i>Bacteroides fragilis</i>	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	<i>Ureaplasma urealyticum</i> , <i>Mycoplasma hominis</i> , <i>Streptococcus agalactiae</i> , <i>Streptococcus anginosus</i> , <i>Prevotella spp</i>	4000	<1	35.6	Stage 2	Stage 3
3	No	3660	21.3	Transabdominal	118.7	Negative	Negative	<i>Staphylococcus hominis</i>	355	<1	21.9	Stage 3	Stage 2
4	No	1160	22.3	Transabdominal	125.5	Gram Negative Bacilli	Positive	<i>Mycoplasma hominis</i> , <i>Fusobacterium nucleatum</i>	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	3.48	Negative	Positive	<i>Mycoplasma hominis</i> , <i>Ureaplasma urealyticum</i>	590	<1	39.6	Stage 1	Stage 1
7	No	258	40.6	C/S	1.9	Negative	Negative	<i>Ureaplasma urealyticum</i> , <i>Staphylococcus haemolyticus</i>	NA	NA	40.6	None	None
8	Yes	18800	40	C/S	47.6	Negative	Positive	<i>Ureaplasma urealyticum</i>	NA	NA	40	Stage 2	Stage 2
9	No	9600	38.1	C/S	101.3	NA	Positive	<i>Mycoplasma hominis</i> , <i>Ureaplasma urealyticum</i>	NA	NA	38.1	Stage 1	Stage 2
10	No	286	39.3	Transabdominal	0.469	NA	Negative	Negative	NA	NA	39.3	None	None

Abbreviations: CS, cesarean section; IL, interleukin; NA, not available; WBC, white blood cell
* Viable Cell Count: Determined with AO/PI on Cellometer 2000 Auto (Nexcelom);