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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 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.

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 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 (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 count[26-31] 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 infection[48-50]. Indeed, amniotic fluid neutrophils are a source of anti-microbial 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 anti-microbial 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 (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 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 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 membranes[86]. 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 leukocytes[31]. The inclusion criteria were (1) singleton gestations, (2) samples without blood contamination, and (3) sufficient amniotic fluid leukocytes (>1 x 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℃) 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/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, 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 criteria[105-108]. 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 RW[108].

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 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, 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 1X 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 x g for 5 min and the supernatant was discarded. The cell pellet was then re-suspended in 5µL 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).

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 x 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 4^0C, the cell pellet was gently washed with 1X 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 ℃ with shaking at 180 rpm. *Escherichia coli* was grown in Luria-Bertani broth (LB, Cat#L7658, Sigma, Saint Louis, MO) at 37 ℃ 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 x g for 5 min 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) at 37℃ with shaking at 180 rpm until a color change (yellow to pink) was observed. The culture broth was then centrifuged at 1,500 x g for 30 min at 4ºC. 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 1X 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 1X PBS containing 20% glycerol (Cat#G1796, TEKnova, Hollister, CA) to an OD_{600} of 0.3 (~1.5 x 10^7/50µL). Fluorescent-labeled bacteria were aliquoted and stored in -80ºC 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 37ºC 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 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 x g for 5 min 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 $2.5x10^5$ 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 37ºC, 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 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-quantificaton 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 method[116]. Briefly, 6 mL of peripheral blood were layered on top of 6 mL of Histopaque

1119 and centrifuged at 800 x 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 (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 $2.5x10^5$ cells/0.5mL for 1 hour at 37ºC 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, 500µL of fresh medium and 50µL of opsonized fluorescent-labeled bacteria were added to the amniotic fluid neutrophils. The culture plate was then centrifuged at 600 x g for 4 minutes and incubated for 1 hour at 37ºC 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 100x/1.40 Oil DIC lens with 1.5x digital zoom.

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

Neutrophils ($2.5x10^5$ cells in 0.5mL of supplemented RPMI medium) were enriched from amniotic fluid samples (Table 2) as described above, mixed with 50µL of

opsonized fluorescent-labeled bacteria in a 1.6 mL Eppendorf tube (Fisher Scientific), and incubated for 1 hour at 37ºC. Next, the tube was centrifuged at 2,300 xg for 5 min and the supernatant was discarded. Electron microscopy fixative was carefully added to the cell pellet. Following fixation for 2 hours at 4^0C, 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 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/mm^3)[26] or viable cell counts (i.e. leukocytes; >100 cells/ mm^3)[56], and 4) 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 *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/mm^3 [26]) or viable cell counts (i.e. leukocytes; >100 cells/mm^3 [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 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 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 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 bacillus[126]. 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 ingested[127-130]. Mycoplasmas can evade phagocytosis by: 1) producing proteases, lipases, phospholipases, and oxygen radicals, which can block the creation or maturation of the phagosome[131-133], 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 infection[129]. 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 membranes[137]. 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 system[138]. 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 phagocytosis[139]. 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 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 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 neutrophils[144-148]. 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 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 *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.

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Disclosure/Conflict of Interest

The authors disclose no conflicts of interest.

REFERENCES

1 Gibbs RS, Romero R, Hillier SL, Eschenbach DA, Sweet RL: A review of premature birth and subclinical infection. *Am J Obstet Gynecol* 1992;**166**:1515-1528.

2 Gomez R, Ghezzi F, Romero R, Munoz H, Tolosa JE, Rojas I: Premature labor and intra-amniotic infection. Clinical aspects and role of the cytokines in diagnosis and pathophysiology. *Clin Perinatol* 1995;**22**:281-342.

3 Goncalves LF, Chaiworapongsa T, Romero R: Intrauterine infection and prematurity. *Ment Retard Dev Disabil Res Rev* 2002;**8**:3-13.

4 Keelan JA, Blumenstein M, Helliwell RJ, Sato TA, Marvin KW, Mitchell MD: Cytokines, prostaglandins and parturition--a review. *Placenta* 2003;**24 Suppl A**:S33-46.

5 Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S: The role of inflammation and infection in preterm birth. *Semin Reprod Med* 2007;**25**:21-39.

6 Bastek JA, Gomez LM, Elovitz MA: The role of inflammation and infection in preterm birth. *Clin Perinatol* 2011;**38**:385-406.

7 Agrawal V, Hirsch E: Intrauterine infection and preterm labor. *Semin Fetal Neonatal Med* 2012;**17**:12-19.

8 Romero R, Chaemsaithong P, Korzeniewski SJ, Tarca AL, Bhatti G, Xu Z, Kusanovic JP, Dong Z, Docheva N, Martinez-Varea A, Yoon BH, Hassan SS, Chaiworapongsa T, Yeo L: Clinical chorioamnionitis at term II: the intra-amniotic inflammatory response. *J Perinat Med* 2016;**44**:5-22.

9 Romero R, Chaemsaithong P, Docheva N, Korzeniewski SJ, Kusanovic JP, Yoon BH, Kim JS, Chaiyasit N, Ahmed AI, Qureshi F, Jacques SM, Kim CJ, Hassan SS, Chaiworapongsa T, Yeo L, Kim YM: Clinical chorioamnionitis at term VI: acute chorioamnionitis and funisitis according to the presence or absence of microorganisms and inflammation in the amniotic cavity. *J Perinat Med* 2016;**44**:33-51.

10 Gibbs RS, Blanco JD, St Clair PJ, Castaneda YS: Quantitative bacteriology of amniotic fluid from women with clinical intraamniotic infection at term. *J Infect Dis* 1982;**145**:1-8.

11 Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, Sabo V, Athanassiadis AP, Hobbins JC: Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *Am J Obstet Gynecol* 1989;**161**:817-824.

12 Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L: A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. *Am J Reprod Immunol* 2014;**71**:330-358.

13 Romero R, Miranda J, Kusanovic JP, Chaiworapongsa T, Chaemsaithong P, Martinez A, Gotsch F, Dong Z, Ahmed AI, Shaman M, Lannaman K, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L, Kim YM: Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using cultivation and molecular techniques. *J Perinat Med* 2015;**43**:19-36.

14 Yoneda N, Yoneda S, Niimi H, Ueno T, Hayashi S, Ito M, Shiozaki A, Urushiyama D, Hata K, Suda W, Hattori M, Kigawa M, Kitajima I, Saito S: Polymicrobial Amniotic Fluid Infection with Mycoplasma/Ureaplasma and Other Bacteria Induces Severe Intra-Amniotic Inflammation Associated with Poor Perinatal Prognosis in Preterm Labor. *Am J Reprod Immunol* 2016;**75**:112-125.

15 Gibbs RS, Dinsmoor MJ, Newton ER, Ramamurthy RS: A randomized trial of intrapartum versus immediate postpartum treatment of women with intra-amniotic infection. *Obstet Gynecol* 1988;**72**:823-828.

16 Gilstrap LC, 3rd, Cox SM: Acute chorioamnionitis. *Obstet Gynecol Clin North Am* 1989;**16**:373-379.

17 Gibbs RS, Duff P: Progress in pathogenesis and management of clinical intraamniotic infection. *Am J Obstet Gynecol* 1991;**164**:1317-1326.

18 Romero R, Chaemsaithong P, Docheva N, Korzeniewski SJ, Tarca AL, Bhatti G, Xu Z, Kusanovic JP, Dong Z, Chaiyasit N, Ahmed AI, Yoon BH, Hassan SS, Chaiworapongsa T, Yeo L: Clinical chorioamnionitis at term IV: the maternal plasma cytokine profile. *J Perinat Med* 2016;**44**:77-98.

19 Gomez R, Romero R, Ghezzi F, Yoon BH, Mazor M, Berry SM: The fetal inflammatory response syndrome. *Am J Obstet Gynecol* 1998;**179**:194-202.

20 Romero R, Chaiworapongsa T, Espinoza J: Micronutrients and intrauterine infection, preterm birth and the fetal inflammatory response syndrome. *J Nutr* 2003;**133**:1668S-1673S.

21 Lee SE, Romero R, Kim CJ, Shim SS, Yoon BH: Funisitis in term pregnancy is associated with microbial invasion of the amniotic cavity and intra-amniotic inflammation. *J Matern Fetal Neonatal Med* 2006;**19**:693-697.

22 Gotsch F, Romero R, Kusanovic JP, Mazaki-Tovi S, Pineles BL, Erez O, Espinoza J, Hassan SS: The fetal inflammatory response syndrome. *Clin Obstet Gynecol* 2007;**50**:652-683.

23 Lee SE, Romero R, Jung H, Park CW, Park JS, Yoon BH: The intensity of the fetal inflammatory response in intraamniotic inflammation with and without microbial invasion of the amniotic cavity. *Am J Obstet Gynecol* 2007;**197**:294 e291-296.

24 Romero R, Chaemsaithong P, Docheva N, Korzeniewski SJ, Tarca AL, Bhatti G, Xu Z, Kusanovic JP, Chaiyasit N, Dong Z, Yoon BH, Hassan SS, Chaiworapongsa T, Yeo L, Kim YM: Clinical chorioamnionitis at term V: umbilical cord plasma cytokine profile in the context of a systemic maternal inflammatory response. *J Perinat Med* 2016;**44**:53-76.

25 Son GH, You YA, Kwon EJ, Lee KY, Kim YJ: Comparative Analysis of Midtrimester Amniotic Fluid Cytokine Levels to Predict Spontaneous Very Preterm Birth in Patients with Cervical Insufficiency. *Am J Reprod Immunol* 2016;**75**:155-161.

26 Romero R, Quintero R, Nores J, Avila C, Mazor M, Hanaoka S, Hagay Z, Merchant L, Hobbins JC: Amniotic fluid white blood cell count: a rapid and simple test to diagnose microbial invasion of the amniotic cavity and predict preterm delivery. *Am J Obstet Gynecol* 1991;**165**:821-830.

27 Romero R, Yoon BH, Mazor M, Gomez R, Diamond MP, Kenney JS, Ramirez M, Fidel PL, Sorokin Y, Cotton D, et al.: The diagnostic and prognostic value of amniotic fluid white blood cell count, glucose, interleukin-6, and gram stain in patients with preterm labor and intact membranes. *Am J Obstet Gynecol* 1993;**169**:805-816.

28 Romero R, Yoon BH, Mazor M, Gomez R, Gonzalez R, Diamond MP, Baumann P, Araneda H, Kenney JS, Cotton DB, et al.: A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count,

interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. *Am J Obstet Gynecol* 1993;**169**:839-851.

29 Gomez R, Romero R, Galasso M, Behnke E, Insunza A, Cotton DB: The value of amniotic fluid interleukin-6, white blood cell count, and gram stain in the diagnosis of microbial invasion of the amniotic cavity in patients at term. *Am J Reprod Immunol* 1994;**32**:200-210.

30 Yoon BH, Yang SH, Jun JK, Park KH, Kim CJ, Romero R: Maternal blood C-reactive protein, white blood cell count, and temperature in preterm labor: a comparison with amniotic fluid white blood cell count. *Obstet Gynecol* 1996;**87**:231-237.

31 Martinez-Varea A, Romero R, Xu Y, Miller D, Ahmed AI, Chaemsaithong P, Chaiyasit N, Yeo L, Shaman M, Lannaman K, Cher B, Hassan SS, Gomez-Lopez N: Clinical chorioamnionitis at term VII: the amniotic fluid cellular immune response. *J Perinat Med* 2016.

32 Romero R, Grivel JC, Tarca AL, Chaemsaithong P, Xu Z, Fitzgerald W, Hassan SS, Chaiworapongsa T, Margolis L: Evidence of perturbations of the cytokine network in preterm labor. *Am J Obstet Gynecol* 2015;**213**:836 e831-836 e818.

33 Romero R, Emamian M, Quintero R, Wan M, Hobbins JC, Mitchell MD: Amniotic fluid prostaglandin levels and intra-amniotic infections. *Lancet* 1986;**1**:1380.

34 Romero R, Emamian M, Wan M, Quintero R, Hobbins JC, Mitchell MD: Prostaglandin concentrations in amniotic fluid of women with intra-amniotic infection and preterm labor. *Am J Obstet Gynecol* 1987;**157**:1461-1467.

35 Romero R, Wu YK, Mazor M, Hobbins JC, Mitchell MD: Amniotic fluid prostaglandin E2 in preterm labor. *Prostaglandins Leukot Essent Fatty Acids* 1988;**34**:141-145.

36 Romero R, Wu YK, Sirtori M, Oyarzun E, Mazor M, Hobbins JC, Mitchell MD: Amniotic fluid concentrations of prostaglandin F2 alpha, 13,14-dihydro-15-keto-prostaglandin F2 alpha (PGFM) and 11-deoxy-13,14-dihydro-15-keto-11, 16-cyclo-prostaglandin E2 (PGEM-LL) in preterm labor. *Prostaglandins* 1989;**37**:149-161.

37 Romero R, Wu YK, Mazor M, Oyarzun E, Hobbins JC, Mitchell MD: Amniotic fluid arachidonate lipoxygenase metabolites in preterm labor. *Prostaglandins Leukot Essent Fatty Acids* 1989;**36**:69-75.

38 Bry K, Hallman M: Prostaglandins, inflammation, and preterm labor. *J Perinatol* 1989;**9**:60-65.

39 Mazor M, Wiznitzer A, Maymon E, Leiberman JR, Cohen A: Changes in amniotic fluid concentrations of prostaglandins E2 and F2 alpha in women with preterm labor. *Isr J Med Sci* 1990;**26**:425-428.

40 Hsu CD, Meaddough E, Aversa K, Hong SF, Lee IS, Bahodo-Singh RO, Lu LC, Copel JA: Dual roles of amniotic fluid nitric oxide and prostaglandin E2 in preterm labor with intra-amniotic infection. *Am J Perinatol* 1998;**15**:683-687.

41 Lee SE, Park IS, Romero R, Yoon BH: Amniotic fluid prostaglandin F2 increases even in sterile amniotic fluid and is an independent predictor of impending delivery in preterm premature rupture of membranes. *J Matern Fetal Neonatal Med* 2009;**22**:880-886.

42 Maddipati KR, Romero R, Chaiworapongsa T, Zhou SL, Xu Z, Tarca AL, Kusanovic JP, Munoz H, Honn KV: Eicosanomic profiling reveals dominance of the epoxygenase pathway in human amniotic fluid at term in spontaneous labor. *FASEB J* 2014;**28**:4835-4846.

43 Bhat G, Williams SM, Saade GR, Menon R: Biomarker interactions are better predictors of spontaneous preterm birth. *Reprod Sci* 2014;**21**:340-350.

44 Menon R, Jones J, Gunst PR, Kacerovsky M, Fortunato SJ, Saade GR, Basraon S: Amniotic fluid metabolomic analysis in spontaneous preterm birth. *Reprod Sci* 2014;**21**:791-803.

45 Park JY, Romero R, Lee J, Chaemsaithong P, Chaiyasit N, Yoon BH: An elevated amniotic fluid prostaglandin F2alpha concentration is associated with intra-amniotic inflammation/infection, and clinical and histologic chorioamnionitis, as well as impending preterm delivery in patients with preterm labor and intact membranes. *J Matern Fetal Neonatal Med* 2016;**29**:2563-2572.

46 Maddipati KR, Romero R, Chaiworapongsa T, Chaemsaithong P, Zhou SL, Xu Z, Tarca AL, Kusanovic JP, Gomez R, Chaiyasit N, Honn KV: Lipidomic analysis of

patients with microbial invasion of the amniotic cavity reveals up-regulation of leukotriene B4. *FASEB J* 2016;**30**:3296-3307.

47 Maddipati KR, Romero R, Chaiworapongsa T, Chaemsaithong P, Zhou SL, Xu Z, Tarca AL, Kusanovic JP, Gomez R, Docheva N, Honn KV: Clinical chorioamnionitis at term: the amniotic fluid fatty acyl lipidome. *J Lipid Res* 2016;**57**:1906-1916.

48 Romero R, Ceska M, Avila C, Mazor M, Behnke E, Lindley I: Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. *Am J Obstet Gynecol* 1991;**165**:813-820.

49 Underwood MA, Gilbert WM, Sherman MP: Amniotic fluid: not just fetal urine anymore. *J Perinatol* 2005;**25**:341-348.

50 Romero R, Kusanovic JP, Espinoza J, Gotsch F, Nhan-Chang CL, Erez O, Kim CJ, Khalek N, Mittal P, Goncalves LF, Schaudinn C, Hassan SS, Costerton JW: What is amniotic fluid 'sludge'? *Ultrasound Obstet Gynecol* 2007;**30**:793-798.

51 Maymon E, Romero R, Chaiworapongsa T, Kim JC, Berman S, Gomez R, Edwin S: Value of amniotic fluid neutrophil collagenase concentrations in preterm premature rupture of membranes. *Am J Obstet Gynecol* 2001;**185**:1143-1148.

52 Helmig BR, Romero R, Espinoza J, Chaiworapongsa T, Bujold E, Gomez R, Ohlsson K, Uldbjerg N: Neutrophil elastase and secretory leukocyte protease inhibitor in prelabor rupture of membranes, parturition and intra-amniotic infection. *J Matern Fetal Neonatal Med* 2002;**12**:237-246.

53 Espinoza J, Chaiworapongsa T, Romero R, Edwin S, Rathnasabapathy C, Gomez R, Bujold E, Camacho N, Kim YM, Hassan S, Blackwell S, Whitty J, Berman S, Redman M, Yoon BH, Sorokin Y: Antimicrobial peptides in amniotic fluid: defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. *J Matern Fetal Neonatal Med* 2003;**13**:2-21.

54 Gravett MG, Novy MJ, Rosenfeld RG, Reddy AP, Jacob T, Turner M, McCormack A, Lapidus JA, Hitti J, Eschenbach DA, Roberts CT, Jr., Nagalla SR:

Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. *JAMA* 2004;**292**:462-469.

55 Soto E, Espinoza J, Nien JK, Kusanovic JP, Erez O, Richani K, Santolaya-Forgas J, Romero R: Human beta-defensin-2: a natural antimicrobial peptide present in amniotic fluid participates in the host response to microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med* 2007;**20**:15-22.

56 Gomez-Lopez N, Romero R, Xu Y, Miller D, Unkel R, Shaman M, Jacques SM, Panaitescu B, Garcia-Flores V, Hassan SS: Neutrophil Extracellular Traps in the Amniotic Cavity of Women with Intra-Amniotic Infection: A New Mechanism of Host Defense. *Reprod Sci* 2016.

57 Gomez-Lopez N, Romero R, Leng Y, Garcia-Flores V, Xu Y, Miller D, Hassan SS: Neutrophil extracellular traps in acute chorioamnionitis: A mechanism of host defense. *Am J Reprod Immunol* 2017;**77**.

58 Romero R, Brody DT, Oyarzun E, Mazor M, Wu YK, Hobbins JC, Durum SK: Infection and labor. III. Interleukin-1: a signal for the onset of parturition. *Am J Obstet Gynecol* 1989;**160**:1117-1123.

59 Romero R, Parvizi ST, Oyarzun E, Mazor M, Wu YK, Avila C, Athanassiadis AP, Mitchell MD: Amniotic fluid interleukin-1 in spontaneous labor at term. *J Reprod Med* 1990;**35**:235-238.

60 Romero R, Mazor M, Brandt F, Sepulveda W, Avila C, Cotton DB, Dinarello CA: Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. *Am J Reprod Immunol* 1992;**27**:117-123.

61 Romero R, Manogue KR, Mitchell MD, Wu YK, Oyarzun E, Hobbins JC, Cerami A: Infection and labor. IV. Cachectin-tumor necrosis factor in the amniotic fluid of women with intraamniotic infection and preterm labor. *Am J Obstet Gynecol* 1989;**161**:336-341.

62 Romero R, Mazor M, Sepulveda W, Avila C, Copeland D, Williams J: Tumor necrosis factor in preterm and term labor. *Am J Obstet Gynecol* 1992;**166**:1576-1587.

63 Cherouny PH, Pankuch GA, Romero R, Botti JJ, Kuhn DC, Demers LM, Appelbaum PC: Neutrophil attractant/activating peptide-1/interleukin-8:

association with histologic chorioamnionitis, preterm delivery, and bioactive amniotic fluid leukoattractants. *Am J Obstet Gynecol* 1993;**169**:1299-1303.

64 Romero R, Shamma F, Avila C, Jimenez C, Callahan R, Nores J, Mazor M, Brekus CA, Hobbins JC: Infection and labor. VI. Prevalence, microbiology, and clinical significance of intraamniotic infection in twin gestations with preterm labor. *Am J Obstet Gynecol* 1990;**163**:757-761.

65 Romero R, Nores J, Mazor M, Sepulveda W, Oyarzun E, Parra M, Insunza A, Montiel F, Behnke E, Cassell GH: Microbial invasion of the amniotic cavity during term labor. Prevalence and clinical significance. *J Reprod Med* 1993;**38**:543-548.

66 Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA: The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. *Obstet Gynecol* 1993;**81**:941-948.

67 Saito S, Kasahara T, Kato Y, Ishihara Y, Ichijo M: Elevation of amniotic fluid interleukin 6 (IL-6), IL-8 and granulocyte colony stimulating factor (G-CSF) in term and preterm parturition. *Cytokine* 1993;**5**:81-88.

68 Halgunset J, Johnsen H, Kjollesdal AM, Qvigstad E, Espevik T, Austgulen R: Cytokine levels in amniotic fluid and inflammatory changes in the placenta from normal deliveries at term. *Eur J Obstet Gynecol Reprod Biol* 1994;**56**:153-160.

69 Arntzen KJ, Kjollesdal AM, Halgunset J, Vatten L, Austgulen R: TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. *J Perinat Med* 1998;**26**:17-26.

70 Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, Kim IO: Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. *Am J Obstet Gynecol* 1997;**177**:19-26.

71 Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI: Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. *Am J Obstet Gynecol* 1997;**177**:825-830.

72 Figueroa R, Garry D, Elimian A, Patel K, Sehgal PB, Tejani N: Evaluation of amniotic fluid cytokines in preterm labor and intact membranes. *J Matern Fetal Neonatal Med* 2005;**18**:241-247.

73 Holst RM, Laurini R, Jacobsson B, Samuelsson E, Savman K, Doverhag C, Wennerholm UB, Hagberg H: Expression of cytokines and chemokines in cervical and amniotic fluid: relationship to histological chorioamnionitis. *J Matern Fetal Neonatal Med* 2007;**20**:885-893.

74 Kacerovsky M, Celec P, Vlkova B, Skogstrand K, Hougaard DM, Cobo T, Jacobsson B: Amniotic fluid protein profiles of intraamniotic inflammatory response to Ureaplasma spp. and other bacteria. *PLoS One* 2013;**8**:e60399.

75 Yoneda S, Shiozaki A, Ito M, Yoneda N, Inada K, Yonezawa R, Kigawa M, Saito S: Accurate Prediction of the Stage of Histological Chorioamnionitis before Delivery by Amniotic Fluid IL-8 Level. *Am J Reprod Immunol* 2015;**73**:568-576.

76 Steinberg BE, Grinstein S: Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death. *Sci STKE* 2007;**2007**:pe11.

77 Brinkmann V, Zychlinsky A: Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* 2007;**5**:577-582.

78 Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. *Science* 2004;**303**:1532-1535.

79 Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A: Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;**176**:231-241.

80 Brinkmann V, Zychlinsky A: Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol* 2012;**198**:773-783.

81 Phillipson M, Kubes P: The neutrophil in vascular inflammation. *Nat Med* 2011;**17**:1381-1390.

82 Tauber AI: Metchnikoff and the phagocytosis theory. *Nat Rev Mol Cell Biol* 2003;**4**:897-901.

83 Gordon S: Phagocytosis: The Legacy of Metchnikoff. *Cell* 2016;**166**:1065-1068.

84 Underhill DM, Gordon S, Imhof BA, Nunez G, Bousso P: Elie Metchnikoff (1845-1916): celebrating 100 years of cellular immunology and beyond. *Nat Rev Immunol* 2016;**16**:651-656.

85 Segal AW, Dorling J, Coade S: Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J Cell Biol* 1980;**85**:42-59.

86 Lee WL, Harrison RE, Grinstein S: Phagocytosis by neutrophils. *Microbes Infect* 2003;**5**:1299-1306.

87 Nordenfelt P, Tapper H: Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* 2011;**90**:271-284.

88 Romero R, Mazor M, Wu YK, Sirtori M, Oyarzun E, Mitchell MD, Hobbins JC: Infection in the pathogenesis of preterm labor. *Semin Perinatol* 1988;**12**:262-279.

89 Romero R, Mazor M: Infection and preterm labor. *Clin Obstet Gynecol* 1988;**31**:553-584.

90 Romero R, Ghidini A, Mazor M, Behnke E: Microbial invasion of the amniotic cavity in premature rupture of membranes. *Clin Obstet Gynecol* 1991;**34**:769-778.

91 Yoon BH, Romero R, Moon JB, Shim SS, Kim M, Kim G, Jun JK: Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Obstet Gynecol* 2001;**185**:1130-1136.

92 Romero R, Chaiworapongsa T, Savasan ZA, Hussein Y, Dong Z, Kusanovic JP, Kim CJ, Hassan SS: Clinical chorioamnionitis is characterized by changes in the expression of the alarmin HMGB1 and one of its receptors, sRAGE. *J Matern Fetal Neonatal Med* 2012;**25**:558-567.

93 Madan I, Romero R, Kusanovic JP, Mittal P, Chaiworapongsa T, Dong Z, Mazaki-Tovi S, Vaisbuch E, Alpay Savasan Z, Yeo L, Kim CJ, Hassan SS: The frequency and clinical significance of intra-amniotic infection and/or inflammation in women with placenta previa and vaginal bleeding: an unexpected observation. *J Perinat Med* 2010;**38**:275-279.

94 Cruciani L, Romero R, Vaisbuch E, Kusanovic JP, Chaiworapongsa T, Mazaki-Tovi S, Mittal P, Ogge G, Gotsch F, Erez O, Kim SK, Dong Z, Pacora P, Lamont

RF, Yeo L, Hassan SS, Di Renzo GC: Pentraxin 3 in amniotic fluid: a novel association with intra-amniotic infection and inflammation. *J Perinat Med* 2010;**38**:161-171.

95 Romero R, Chaiworapongsa T, Alpay Savasan Z, Xu Y, Hussein Y, Dong Z, Kusanovic JP, Kim CJ, Hassan SS: Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. *J Matern Fetal Neonatal Med* 2011;**24**:1444-1455.

96 Gervasi MT, Romero R, Bracalente G, Erez O, Dong Z, Hassan SS, Yeo L, Yoon BH, Chaiworapongsa T: Midtrimester amniotic fluid concentrations of interleukin-6 and interferon-gamma-inducible protein-10: evidence for heterogeneity of intra-amniotic inflammation and associations with spontaneous early (<32 weeks) and late (>32 weeks) preterm delivery. *J Perinat Med* 2012;**40**:329-343.

97 Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L, Kim YM: Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. *J Matern Fetal Neonatal Med* 2014:1-17.

98 Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Yeo L: Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol* 2014;**72**:458-474.

99 Combs CA, Gravett M, Garite TJ, Hickok DE, Lapidus J, Porreco R, Rael J, Grove T, Morgan TK, Clewell W, Miller H, Luthy D, Pereira L, Nageotte M, Robilio PA, Fortunato S, Simhan H, Baxter JK, Amon E, Franco A, Trofatter K, Heyborne K, ProteoGenix/Obstetrix Collaborative Research N: Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *Am J Obstet Gynecol* 2014;**210**:125 e121-125 e115.

100 Kacerovsky M, Musilova I, Andrys C, Hornychova H, Pliskova L, Kostal M, Jacobsson B: Prelabor rupture of membranes between 34 and 37 weeks: the intraamniotic inflammatory response and neonatal outcomes. *Am J Obstet Gynecol* 2014;**210**:325 e321-325 e310.

101 Romero R, Miranda J, Chaemsaithong P, Chaiworapongsa T, Kusanovic JP, Dong Z, Ahmed AI, Shaman M, Lannaman K, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L, Kim YM: Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med* 2015;**28**:1394-1409.

102 Chaemsaithong P, Romero R, Korzeniewski SJ, Dong Z, Yeo L, Hassan SS, Kim YM, Yoon BH, Chaiworapongsa T: A point of care test for the determination of amniotic fluid interleukin-6 and the chemokine CXCL-10/IP-10. *J Matern Fetal Neonatal Med* 2015;**28**:1510-1519.

103 Chaemsaithong P, Romero R, Korzeniewski SJ, Martinez-Varea A, Dong Z, Yoon BH, Hassan SS, Chaiworapongsa T, Yeo L: A point of care test for interleukin-6 in amniotic fluid in preterm prelabor rupture of membranes: a step toward the early treatment of acute intra-amniotic inflammation/infection. *J Matern Fetal Neonatal Med* 2016;**29**:360-367.

104 Chaemsaithong P, Romero R, Korzeniewski SJ, Martinez-Varea A, Dong Z, Yoon BH, Hassan SS, Chaiworapongsa T, Yeo L: A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. *J Matern Fetal Neonatal Med* 2016;**29**:349-359.

105 Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C, Society for Pediatric Pathology PSAFINC: Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. *Pediatr Dev Pathol* 2003;**6**:435-448.

106 Redline RW: Inflammatory responses in the placenta and umbilical cord. *Semin Fetal Neonatal Med* 2006;**11**:296-301.

107 Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM: Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. *Am J Obstet Gynecol* 2015;**213**:S29-52.

108 Redline RW: Classification of placental lesions. *Am J Obstet Gynecol* 2015;**213**:S21-28.

109 Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH, Syn HC: Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory

lesions of preterm placenta and prediction of perinatal morbidity. *Am J Obstet Gynecol* 1995;**172**:960-970.

110 Yoon BH, Romero R, Lim JH, Shim SS, Hong JS, Shim JY, Jun JK: The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. *Am J Obstet Gynecol* 2003;**189**:919-924.

111 DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman DA: Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008;**3**:e3056.

112 DiGiulio DB, Gervasi MT, Romero R, Vaisbuch E, Mazaki-Tovi S, Kusanovic JP, Seok KS, Gomez R, Mittal P, Gotsch F, Chaiworapongsa T, Oyarzun E, Kim CJ, Relman DA: Microbial invasion of the amniotic cavity in pregnancies with small-for-gestational-age fetuses. *J Perinat Med* 2010;**38**:495-502.

113 Romero R, Jimenez C, Lohda AK, Nores J, Hanaoka S, Avila C, Callahan R, Mazor M, Hobbins JC, Diamond MP: Amniotic fluid glucose concentration: a rapid and simple method for the detection of intraamniotic infection in preterm labor. *Am J Obstet Gynecol* 1990;**163**:968-974.

114 Romero R, Emamian M, Quintero R, Wan M, Hobbins JC, Mazor M, Edberg S: The value and limitations of the Gram stain examination in the diagnosis of intraamniotic infection. *Am J Obstet Gynecol* 1988;**159**:114-119.

115 Kim MJ, Romero R, Gervasi MT, Kim JS, Yoo W, Lee DC, Mittal P, Erez O, Kusanovic JP, Hassan SS, Kim CJ: Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. *Lab Invest* 2009;**89**:924-936.

116 Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A: Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp* 2010.

117 Mi Lee S, Romero R, Lee KA, Jin Yang H, Joon Oh K, Park CW, Yoon BH: The frequency and risk factors of funisitis and histologic chorioamnionitis in pregnant women at term who delivered after the spontaneous onset of labor. *J Matern Fetal Neonatal Med* 2011;**24**:37-42.

118 Yoon BH, Romero R, Park JS, Kim M, Oh SY, Kim CJ, Jun JK: The relationship among inflammatory lesions of the umbilical cord (funisitis), umbilical cord plasma interleukin 6 concentration, amniotic fluid infection, and neonatal sepsis. *Am J Obstet Gynecol* 2000;**183**:1124-1129.

119 Park JS, Romero R, Yoon BH, Moon JB, Oh SY, Han SY, Ko EM: The relationship between amniotic fluid matrix metalloproteinase-8 and funisitis. *Am J Obstet Gynecol* 2001;**185**:1156-1161.

120 Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, Ghezzi F, Berry SM, Qureshi F, Jacques SM, Kim JC, Kadar N, Romero R: Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. *J Matern Fetal Neonatal Med* 2002;**11**:18-25.

121 Park CW, Lee SM, Park JS, Jun JK, Romero R, Yoon BH: The antenatal identification of funisitis with a rapid MMP-8 bedside test. *J Perinat Med* 2008;**36**:497-502.

122 Oh KJ, Kim SM, Hong JS, Maymon E, Erez O, Panaitescu B, Gomez-Lopez N, Romero R, Yoon BH: Twenty-four percent of patients with clinical chorioamnionitis in preterm gestations have no evidence of either culture-proven intraamniotic infection or intraamniotic inflammation. *Am J Obstet Gynecol* 2017.

123 Gibbs RS, Weiner MH, Walmer K, St Clair PJ: Microbiologic and serologic studies of Gardnerella vaginalis in intra-amniotic infection. *Obstet Gynecol* 1987;**70**:187-190.

124 Henneke P, Berner R: Interaction of neonatal phagocytes with group B streptococcus: recognition and response. *Infect Immun* 2006;**74**:3085-3095.

125 Carlin AF, Chang YC, Areschoug T, Lindahl G, Hurtado-Ziola N, King CC, Varki A, Nizet V: Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *J Exp Med* 2009;**206**:1691-1699.

126 Lewis MA, Milligan SG, MacFarlane TW, Carmichael FA: Phagocytosis of bacterial strains isolated from acute dentoalveolar abscess. *J Med Microbiol* 1993;**38**:151-154.

127 Simberkoff MS, Elsbach P: The interaction in vitro between polymorphonuclear leukocytes and mycoplasma. *J Exp Med* 1971;**134**:1417-1430.

128 Krausse R, Ullmann U, Wagener C: In vitro influence of Mycoplasma species on the stimulation of human polymorphonuclear granulocytes. *Zentralbl Bakteriol Mikrobiol Hyg A* 1988;**270**:228-236.

129 Webster AD, Furr PM, Hughes-Jones NC, Gorick BD, Taylor-Robinson D: Critical dependence on antibody for defence against mycoplasmas. *Clin Exp Immunol* 1988;**71**:383-387.

130 Marshall AJ, Miles RJ, Richards L: The phagocytosis of mycoplasmas. *J Med Microbiol* 1995;**43**:239-250.

131 De Silva NS, Quinn PA: Endogenous activity of phospholipases A and C in Ureaplasma urealyticum. *J Clin Microbiol* 1986;**23**:354-359.

132 Bhandari S, Asnani PJ: Characterization of phospholipase A2 of mycoplasma species. *Folia Microbiol (Praha)* 1989;**34**:294-301.

133 Almagor M, Kahane I, Yatziv S: Role of superoxide anion in host cell injury induced by mycoplasma pneumoniae infection. A study in normal and trisomy 21 cells. *J Clin Invest* 1984;**73**:842-847.

134 Niederman R, Brunkhorst B, Smith S, Weinreb RN, Ryder MI: Ammonia as a potential mediator of adult human periodontal infection: inhibition of neutrophil function. *Arch Oral Biol* 1990;**35 Suppl**:205S-209S.

135 Brunkhorst B, Niederman R: Ammonium decreases human polymorphonuclear leukocyte cytoskeletal actin. *Infect Immun* 1991;**59**:1378-1386.

136 Cole BC, Ward JR: Interaction of Mycoplasma arthritidis and other mycoplasmas with murine peritoneal macrophages. *Infect Immun* 1973;**7**:691-699.

137 Noda-Nicolau NM, Polettini J, Peltier MR, da Silva MG, Menon R: Combinations and loads of bacteria affect the cytokine production by fetal membranes: An in vitro study. *Am J Reprod Immunol* 2016;**76**:504-511.

138 Easmon CS, Clark L, Crane JP, Green R: Phagocytosis and killing of Gardnerella vaginalis by human neutrophils. *J Clin Pathol* 1985;**38**:747-749.

139 Burns SM, Hull SI: Loss of resistance to ingestion and phagocytic killing by O(-) and K(-) mutants of a uropathogenic Escherichia coli O75:K5 strain. *Infect Immun* 1999;**67**:3757-3762.

140 Nazareth H, Genagon SA, Russo TA: Extraintestinal pathogenic Escherichia coli survives within neutrophils. *Infect Immun* 2007;**75**:2776-2785.

141 Sampson JE, Theve RP, Blatman RN, Shipp TD, Bianchi DW, Ward BE, Jack RM: Fetal origin of amniotic fluid polymorphonuclear leukocytes. *Am J Obstet Gynecol* 1997;**176**:77-81.

142 Macias AE, Wong SW, Sadowsky DW, Luetjens CM, Axthelm MK, Gravett MG, Haluska GJ, Novy MJ: Maternal or fetal origin of rhesus monkey (Macaca mulatta) amniotic fluid leukocytes can be identified by polymerase chain reaction using the zinc finger Y gene. *Am J Primatol* 2001;**55**:159-170.

143 Lee SD, Kim MR, Hwang PG, Shim SS, Yoon BH, Kim CJ: Chorionic plate vessels as an origin of amniotic fluid neutrophils. *Pathol Int* 2004;**54**:516-522.

144 Pahwa SG, Pahwa R, Grimes E, Smithwick E: Cellular and humoral components of monocyte and neutrophil chemotaxis in cord blood. *Pediatr Res* 1977;**11**:677-680.

145 Anderson DC, Abbassi O, Kishimoto TK, Koenig JM, McIntire LV, Smith CW: Diminished lectin-, epidermal growth factor-, complement binding domain-cell adhesion molecule-1 on neonatal neutrophils underlies their impaired CD18-independent adhesion to endothelial cells in vitro. *J Immunol* 1991;**146**:3372-3379.

146 Mariscalco MM, Tcharmtchi MH, Smith CW: P-Selectin support of neonatal neutrophil adherence under flow: contribution of L-selectin, LFA-1, and ligand(s) for P-selectin. *Blood* 1998;**91**:4776-4785.

147 Levy O, Martin S, Eichenwald E, Ganz T, Valore E, Carroll SF, Lee K, Goldmann D, Thorne GM: Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein. *Pediatrics* 1999;**104**:1327-1333.

148 Levy O: Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* 2007;**7**:379-390.

149 Marodi L, Leijh PC, van Furth R: Characteristics and functional capacities of human cord blood granulocytes and monocytes. *Pediatr Res* 1984;**18**:1127-1131.

150 McEvoy LT, Zakem-Cloud H, Tosi MF: Total cell content of CR3 (CD11b/CD18) and LFA-1 (CD11a/CD18) in neonatal neutrophils: relationship to gestational age. *Blood* 1996;**87**:3929-3933.

151 Kallman J, Schollin J, Schalen C, Erlandsson A, Kihlstrom E: Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates. *Arch Dis Child Fetal Neonatal Ed* 1998;**78**:F46-50.

152 Henneke P, Osmers I, Bauer K, Lamping N, Versmold HT, Schumann RR: Impaired CD14-dependent and independent response of polymorphonuclear leukocytes in preterm infants. *J Perinat Med* 2003;**31**:176-183.

Figure Legends

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, 8,000X; sample 2, 2,000X; sample 3, 2,500X; sample 4, 15,000X; sample 5, 2,500X; and sample 6, 4,000X). 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 10,000X. B) A scanning electron microscopy image of amniotic fluid neutrophils and *Streptococcus agalactiae* (red arrow) prior to phagocytosis. Magnification 6,000X. 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 630X. D) A transmission electron microscopy image of a neutrophil engulfing *Streptococcus agalactiae*. Magnification 2,500X. 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 10,000X. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Ureaplasma urealyticum* (red arrows) prior to phagocytosis. Magnification 5,000X. 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 630X. D) A transmission electron microscopy image of a neutrophil engulfing *Ureaplasma urealyticum*. Magnification 2,500X. 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 5,000X. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Gardnerella vaginalis* (red arrow) prior to phagocytosis. Magnification 7,500X. 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 630X. D) A transmission electron microscopy image of a neutrophil engulfing *Gardnerella vaginalis*. Magnification 3,000X. 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 5,000X. B) A scanning electron microscopy image of an amniotic fluid neutrophil and *Escherichia coli* (red arrow) prior to phagocytosis. Magnification 7,500X. 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 630X. D) A transmission electron microscopy image of a neutrophil engulfing *Escherichia coli*. Magnification 1,200X. 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.

Supplementary Figures

Figure 1: Peripheral neutrophils without phagocytized bacteria from a healthy individual. Transmission electron microscopy images were captured at a 3,000X magnification. Nuclei and organelles are seen; yet, bacteria are absent.

Video Legends

Video 1. Live imaging of *ex vivo* phagocytosis of *Streptococcus agalactiae* by amniotic fluid neutrophils. Confocal time series of amniotic fluid neutrophils phagocytizing *Streptococcus agalactiae* (white arrow) were recorded with a frame size of 512 × 512 pixels at 7.75-s time intervals from 5 to 20 mins.

Video 2. Live imaging of *ex vivo* phagocytosis of *Ureaplasma urealyticum* by amniotic fluid neutrophils. Confocal time series of amniotic fluid neutrophils phagocytizing *Ureaplasma urealyticum* (white arrow) were recorded with a frame size of 512 × 512 pixels at 7.75-s time intervals from 5 to 20 mins.

Video 3. Live imaging of *ex vivo* phagocytosis of *Gardnerella vaginalis* by amniotic fluid neutrophils. Confocal time series of amniotic fluid neutrophils phagocytizing *Gardnerella vaginalis* (white arrow) were recorded with a frame size of 512 × 512 pixels at 7.75-s time intervals from 5 to 20 mins.

Video 4. Live imaging of *ex vivo* phagocytosis of *Escherichia coli* by amniotic fluid neutrophils. Confocal time series of amniotic fluid neutrophils phagocytizing *Escherichia coli* (white arrow) were recorded with a frame size of 512 × 512 pixels at 7.75-s time intervals from 5 to 20 mins.

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	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	18800	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

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

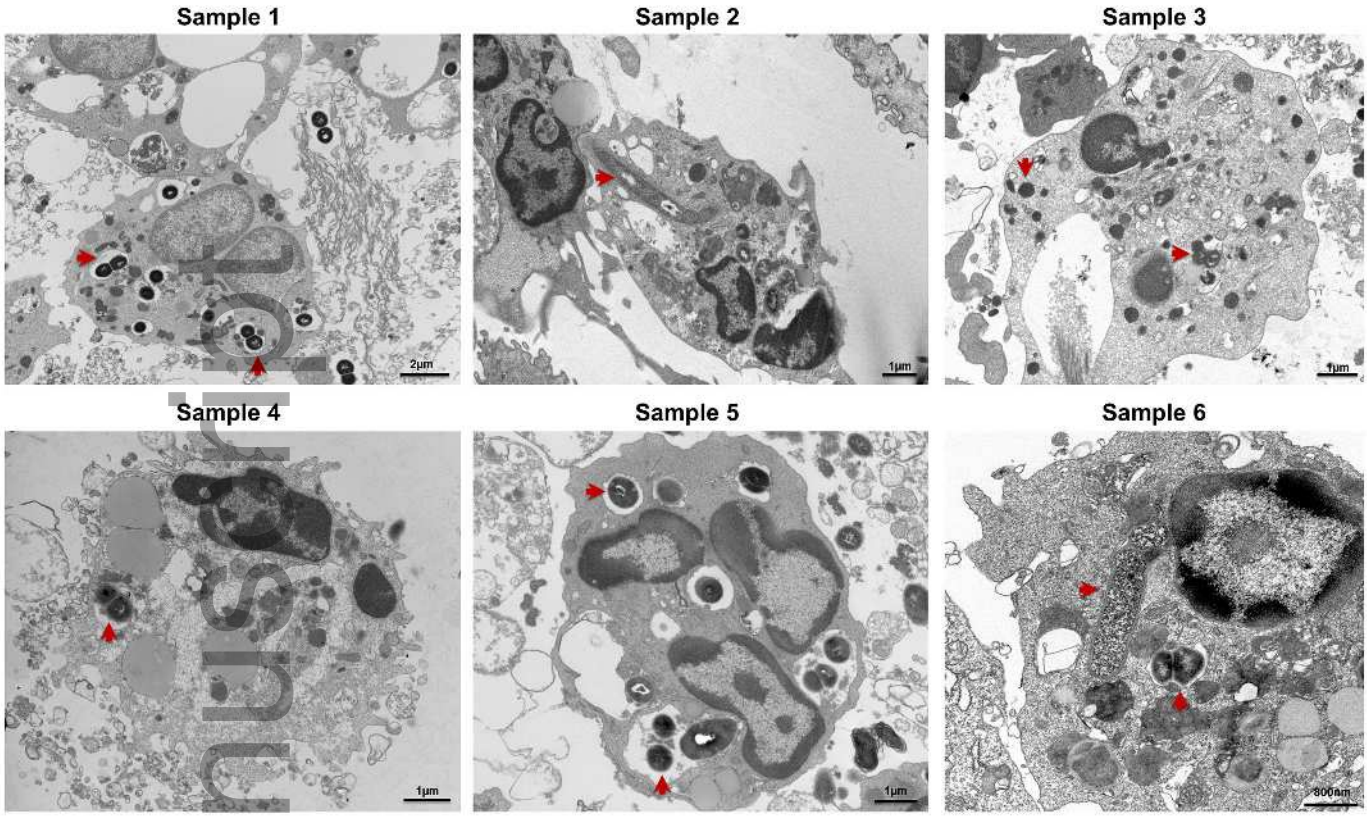
Sample	Clinical Chorioamnionitis	Viable Cell Count* (cells/mm^3)	Gestational Age at Amniocentesis	Collection Method of Amniotic Fluid	IL-6 (ng/mL)	Gram Stain	Bacterial Live/Dead Staining	Amniotic Fluid Culture	WBC (cells/mm^3)	Glucose (mg/dL)	Gestational Age at delivery	Placental Pathology	
												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	3.48	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	18800	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*,	NA	NA	38.1	Stage 1	Stage 2

								Ureaplasma urealyticum					
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);

Table 2. Clinical characteristics of amniotic fluid samples utilized for *ex vivo* phagocytosis assays.



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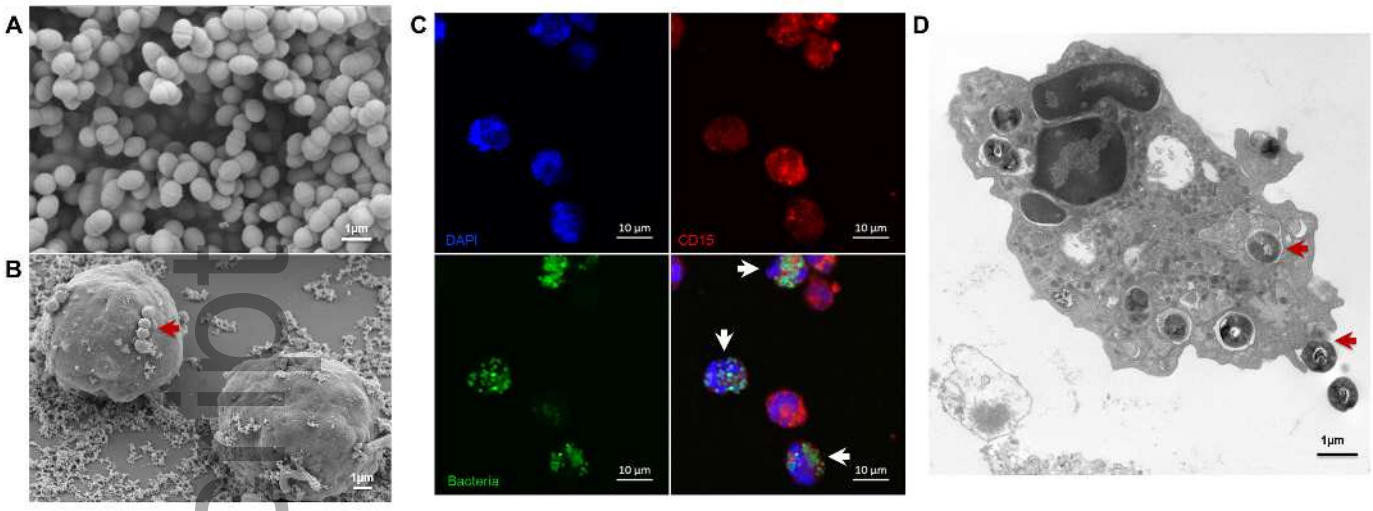

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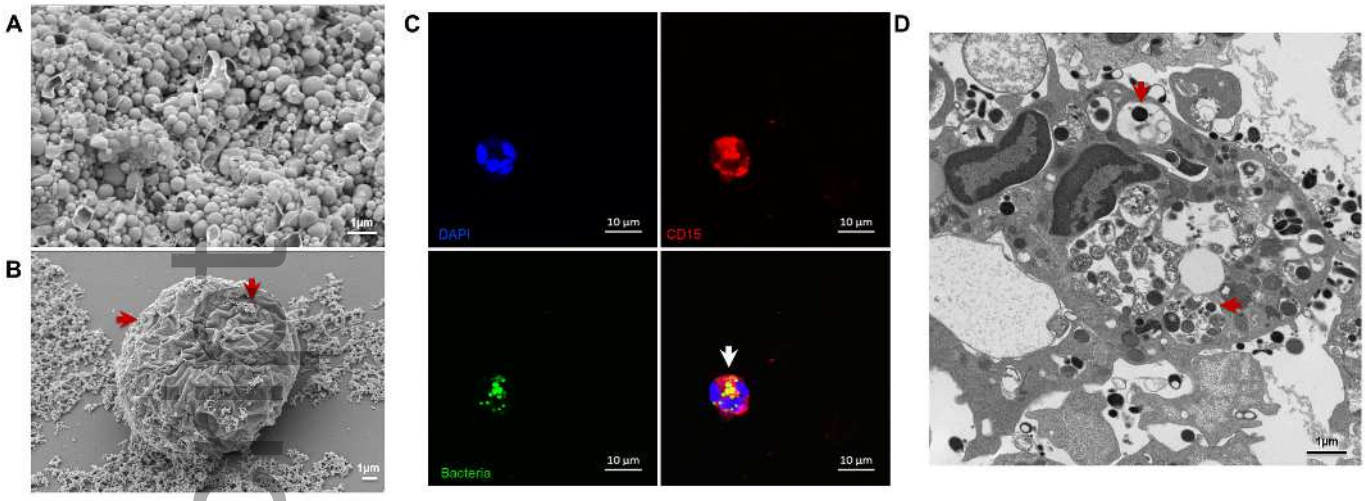

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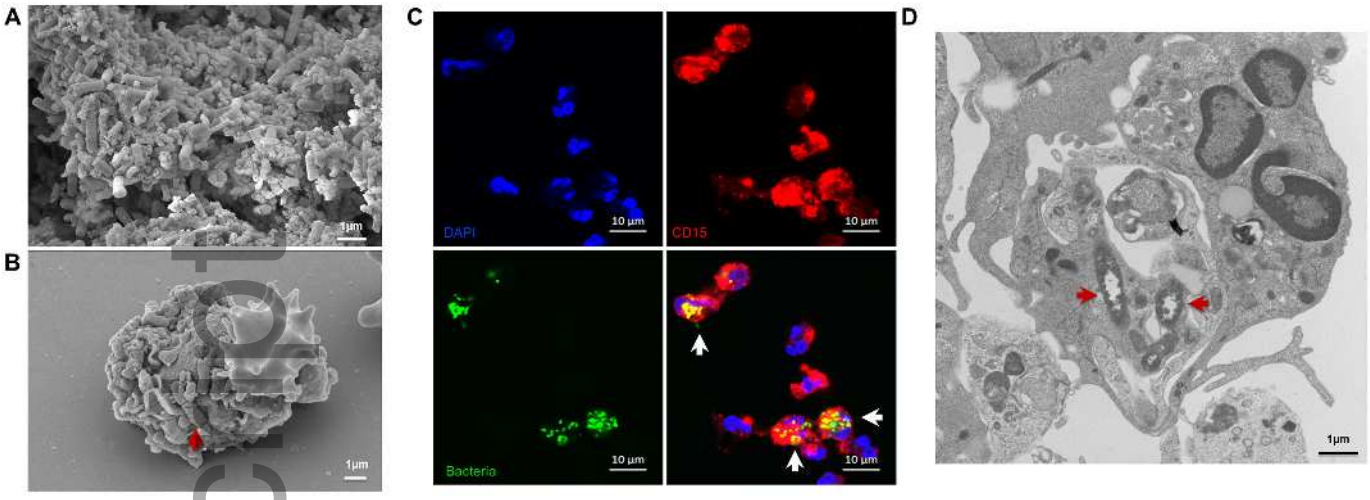

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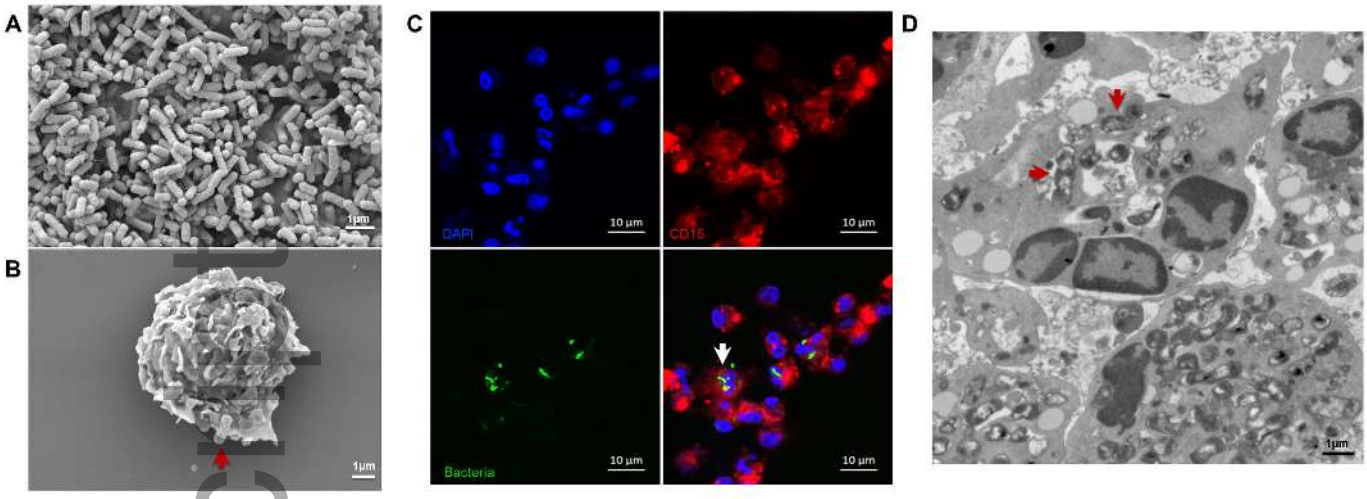

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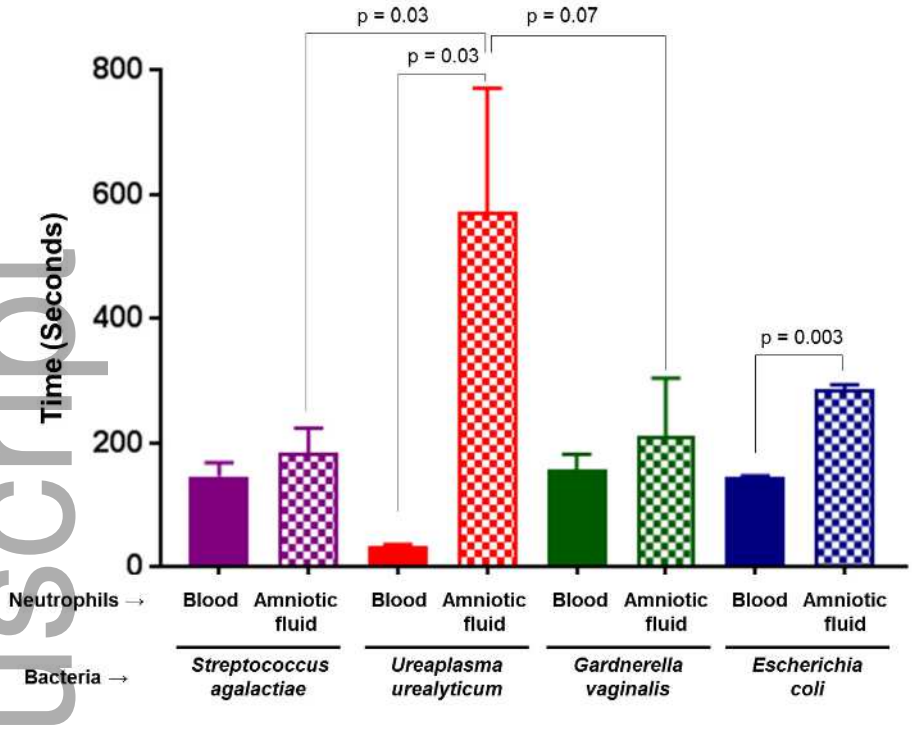

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