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Neutrophil Extracellular Traps in the Amniotic Cavity of Women with Intra-Amniotic Infection: A New Mechanism of Host Defense

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Abstract

Objective: Neutrophil extracellular traps (NETs) control microbial infections through their antimicrobial activities attributed to DNA, histones, granules, and cytoplasmic proteins (eg, elastase). Intra-amniotic infection is characterized by the influx of neutrophils into the amniotic cavity; therefore, the aim of this study was to determine whether amniotic fluid neutrophils form NETs in this inflammatory process. **Methods:** Amniotic fluid samples from women with intra-amniotic infection (n = 15) were stained for bacteria detection using fluorescent dyes. Amniotic fluid neutrophils were purified by filtration. As controls, neutrophils from maternal blood samples (n = 3) were isolated by density gradients. Isolated neutrophils were plated onto glass cover slips for culture with and without 100 nM of phorbol-12-myristate-13-acetate (PMA). NET formation was assessed by 4',6-diamidino-2-phenylindole (DAPI) staining and scanning electron microscopy. Different stages of NET formation were visualized using antibodies against elastase and histone H3, in combination with DAPI staining, by confocal microscopy. Finally, maternal or neonatal neutrophils were added to amniotic fluid samples from women without intra-amniotic infection (n = 4), and NET formation was evaluated by DAPI staining. **Results:** (1) NETs were present in the amniotic fluid of women with intra-amniotic infection; (2) all of the amniotic fluid samples had detectable live and dead bacteria associated with the presence of NETs; (3) in contrast to neutrophils from the maternal circulation, amniotic fluid neutrophils did not require PMA stimulation to form NETs; (4) different stages of NET formation were observed by co-localizing elastase, histone H3, and DNA in amniotic fluid neutrophils; and (5) neither maternal nor neonatal neutrophils form NETs in the amniotic fluid of women without intra-amniotic infection. **Conclusion:** NETs are detectable in the amniotic fluid of women with intra-amniotic infection.

Keywords

cytokines, fetal inflammatory response, fever, funisitis, inflammation, intra-amniotic inflammation, labor, microbial invasion of the amniotic cavity (MIAC), neutrophil, parturition, pregnancy, preterm birth, preterm labor

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Introduction

Intra-amniotic inflammation can be due to microorganisms (ie, intra-amniotic infection) or danger signals derived from necrosis and cellular stress (ie, sterile intra-amniotic inflammation).[1-6] Intra-amniotic infection is frequently caused by *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and other commensal organisms found in the lower genital tract.[7-10] Microbial invasion of the amniotic cavity (MIAC) can elicit local and systemic inflammatory responses.[11-15] Systemic maternal inflammation results in clinical chorioamnionitis, which refers to the presence of maternal fever associated with clinical signs (ie, foul-smelling discharge, uterine tenderness, and maternal and fetal tachycardia) as well as laboratory abnormalities such as leukocytosis.[16,17]

In humans, intra-amniotic infection is associated with a local inflammatory response,[18] which is characterized by an increased amniotic fluid white blood cell (WBC) count[19-23] and elevated concentrations of inflammatory mediators, such as cytokines[18,24] and lipids (eg, prostaglandins).[25-38] In nonhuman primates, intra-amniotic infection also results in a local inflammatory response.[39-48] Neutrophils are the most abundant leukocytes in the amniotic fluid in cases of intra-amniotic infection.[19] These innate immune cells seem to be fetal[49] and originate from the chorionic plate;[50] yet, further evidence is required to prove their fetal origin using genetic fingerprinting. Recently, using immunophenotyping, we demonstrated that amniotic fluid neutrophils express proinflammatory cytokines such as tumor necrosis factor-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, interleukin (IL)-1α, IL-1β, and IL-8.[51] These cytokines and chemokines have been implicated in the processes responsible for term and preterm parturition.[24,52-74]

Neutrophils are the first line of defense against invading pathogens and possess an arsenal of weapons utilized in the elimination of microbes.[75] These innate immune cells are primarily phagocytes, capable of enveloping and killing microbes through the initiation of NADPH oxidase activity, which leads to the release of reactive oxygen species such as peroxide, superoxide, and hydroxyl radical.[76,77] Neutrophils carry granules filled with enzymes such as myeloperoxidase, cathepsin G, elastase, and proteinase 3, which can be injected into the phagosome or released externally, leading to bacterial killing.[75,76]

Neutrophils can also undergo a specialized cell death termed neutrophil extracellular traps (NETs),[78,79] which represents the final containment effort of a neutrophil to lyse pathogens.[80] NETs are web-like structures composed of DNA, histones, and antimicrobial products such as neutrophil elastase.[78-80] These traps eliminate microbes through their biochemical components.[81] Histones (~ 50% of the extracellular trap[82]) use their cationic properties to kill bacteria by permeabilizing microbial membranes.[81] Extracellular DNA also has microbicidal properties as it is a powerful chelator of divalent cations.[83] Therefore, the main function of NETs in immune host defense is to prevent infection by trapping and killing bacteria. The aim of this study was to investigate whether neutrophils in the amniotic cavity of women with intra-amniotic infection are capable of forming NETs.

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 (Tables 1 and 2). Patients were enrolled at Hutzel Women's Hospital of the Detroit Medical Center (Detroit, Michigan). The inclusion criteria were (1) singleton gestations and (2) sufficient viable cells ($>1 \times 10^5$ cells/mL) in amniotic fluid samples. Viable cell numbers were determined using an automatic cell counter (Cellometer Auto 2000; Nexcelom Bioscience, Lawrence, Massachusetts).

Maternal and neonatal data were obtained by retrospective clinical chart review. The information retrieved included the following: use of epidural analgesia, intrapartum antibiotic administration, membrane status at the time of amniocentesis (intact or ruptured), and mode of delivery. Patients with the diagnosis of clinical chorioamnionitis (see diagnostic criteria in the subsequent section) were counseled by their treating physicians about the potential value of knowing the precise microorganism(s) involved in the suspected infection. Further management of these patients was at the discretion of the attending physician.

All 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, Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services (NICHD/NIH/DHHS).

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 if the estimation was inconsistent with menstrual dating. Clinical chorioamnionitis was diagnosed by the presence of maternal fever accompanied by 2 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).[11,84-86] Labor at term was defined as the presence of regular uterine contractions with a frequency of at least 1 every 10 minutes and cervical change after 37 weeks of gestation. Preterm labor was diagnosed by the presence of regular uterine contractions (at least 3 in 30 minutes) and documented cervical changes in patients with a gestational age between 20 and 36 (6/7) weeks.

Microbial invasion of the amniotic cavity was defined as a positive amniotic fluid culture.[87-91] Intra-amniotic inflammation was diagnosed when the IL-6 concentration in amniotic

Table 1. Demographic and Clinical Characteristics of Samples Used in This Study.

	Term Delivery (n = 9)	Preterm Delivery (n = 6)	*P* Value
Maternal age, years; median (IQR)[a]	21 (19-22)	25.5 (24.3-32.8)	.028
Body mass index, kg/m^2; median (IQR)[a]	25.6 (23.7-30.2)	33.3 (27.3-35.8)	NS
Gestational age at delivery, week; median (IQR)[a]	39.9 (39.3-40.3)	24 (23.3-29.3)	.001
Birth weight, g; median (IQR)[a]	3440 (3385-3635)	630 (552.5-1435)	.01
Race, n (%)[b]			NS
African American	8 (88.9)	6 (100)	
Caucasian	0 (0)	0 (0)	
Hispanic	0 (0)	0 (0)	
Asian	1 (11.1)	0 (0)	
Other	0 (0)	0 (0)	
Labor, n (%)[b]	9 (100)	5 (83.3)	NS
Primiparity, n (%)[b]	5 (55.6)	1 (16.7)	NS
Cesarean section, n (%)[b]	6 (66.7)	2 (33.3)	NS
Acute maternal inflammatory response, n (%)[b]			
Stage 1 (acute subchorionitis)	1 (11.1)	0 (0)	NS
Stage 2 (acute chorioamnionitis)	4 (44.4)	1 (16.7)	NS
Stage 3 (acute necrotizing chorioamnionitis)	4 (44.4)	5 (83.3)	NS
Acute fetal inflammatory response, n(%)[b]			
Stage 1 (acute phlebitis/chorionic vasculitis)	4 (44.4)	1 (16.7)	NS
Stage 2 (acute arteritis)	5 (55.6)	3 (50)	NS
Stage 3 (necrotizing funisitis)	0 (0)	2 (33.3)	NS

Abbreviation: IQR, interquartile range.
[a]Wilcoxon rank sum test.
[b]Fisher's exact test.

fluid was $\geq$ 2.6 ng/mL.[92,93] Intra-amniotic infection was defined as the presence of MIAC with intra-amniotic inflammation.[3-6,10,92-102] Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes,[103-106] whereas acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly, using previously described criteria.[103,105,107-110]

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.[10,19,111-114] Shortly after collection, the WBC count in amniotic fluid samples was determined using a hemocytometer chamber, according to previously described methods.[19] Glucose concentration[115] was also determined and Gram stain[116] was performed in amniotic fluid samples. Cultures, WBC count, glucose concentration, and Gram Stain were not performed in those amniotic fluid samples collected during cesarean section, as these samples were collected for research purposes only. However, both IL-6 concentration and the presence of bacteria (bacterial live/dead staining) were assessed in all of the amniotic fluid samples.

Determination of IL-6 in Amniotic Fluid

Interleukin-6 concentrations in amniotic fluid samples were determined using a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, Minnesota). IL-6 concentrations were determined by interpolation from the standard curve. The interassay and intraassay 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 NETs by DNA Staining

NETs were discovered when isolated peripheral neutrophils were stimulated with phorbol-12-myristate-13-acetate (PMA) in vitro.[78] Therefore, we used maternal peripheral neutrophils as positive controls. Peripheral blood samples were collected by venipuncture into EDTA-containing tubes from pregnant women at term in the absence of labor (n = 3). Neutrophils were isolated using the density gradient reagent Histopaque 1119 (Sigma-Aldrich; St. Louis, Missouri), according to the manufacturer's instructions and a previously published method.[117] Briefly, 6 mL of peripheral blood were layered on top of 6 mL of Histopaque 1119 and centrifuged at 800*g* for 20 minutes with no break at room temperature. Neutrophils were collected from the lower phase of the gradient after the peripheral blood mononuclear cell band was discarded. The collected neutrophils were further purified using a gradient composed of 85%, 80%, 75%, 70%, and 65% Percoll (GE Healthcare Life Sciences; Uppsala, Sweden) and washed with 1X PBS (Life Technologies; Grand Island, New York). Purified neutrophils

Table 2. Clinical Characteristics of Amniotic Fluid Samples Utilized in This Study.

Sample	Clinical Chorioamnionitis	Viable Cell Count[a] (cells/mm^3)	Gestational Age at Amniocentesis	Collection Method of Amniotic Fluid	IL-6 (ng/mL)	Gram Stain	Amniotic Fluid Culture	WBC Count (cells/mm^3)	Glucose (mg/dL)	Gestational Age at Delivery	Bacterial Live/Dead Staining	Delivery Method
1	Yes	1080	39.9	Transabdominal	87.0	Negative	*Ureaplasma* subspecies, *Streptococcus anginosus*	7183	<1	39.9	Positive	C/S
2	Yes	3570	40.1	Transabdominal	32.3	Gram-positive cocci, Gram-variable bacilli	*Ureaplasma* subspecies, *Peptostreptococcus* subspecies, *Viridans streptococci, Enterococcus faecalis*	3000	<1	40.3	Positive	C/S
3	Yes	927	39.7	Transabdominal	30.3	Gram-positive cocci, Gram-positive bacilli	*Ureaplasma* subspecies, *S agalactiae*	530	<1	39.7	Positive	Vaginal
4	Yes	1040	40.9	Transabdominal	113.5	Gram-negative bacilli	*Ureaplasma* subspecies, *Mycoplasma* subspecies, *Fusobacterium* subspecies	878	<1	40.9	Positive	Vaginal
5	Yes	1100	37.7	Transabdominal	16.9	Negative	*Lactobacillus* subspecies, *Peptostreptococcus* subspecies, *Gardnerella vaginalis, S anginosus*	900	7	37.7	Positive	Vaginal
6	Yes	996	22.7	Transabdominal	334.9	Negative	*Fusobacterium* subspecies	463	<1	23.3	Positive	C/S
7	Yes	697	24.6	Transabdominal	259.4	Gram-negative bacilli	*Escherichia coli*	609	<1	24.6	Positive	Vaginal
8	No	1430	30.7	Transabdominal	60.0	Negative	*Ureaplasma* subspecies, *Mycoplasma* subspecies	1400	12	30.9	Positive	Vaginal
9	Yes	2220	36.6	Transabdominal	8.1	Gram-positive cocci	*S agalactiae*	310	<1	36.7	Positive	Vaginal
10	Yes	508	23.1	Transabdominal	121.0	Gram-negative bacilli	*Ureaplasma* subspecies, *G vaginalis, Bacteroides ureolyticus*	750	<1	23.4	Positive	C/S
11	Yes	468	20.7	Transabdominal	122.1	Gram-positive cocci	*Ureaplasma* subspecies, *S mitis, Eikenella corrodens*	1150	17	20.7	Positive	Vaginal
12	Yes	11 100	39.3	C/S	106.9	NA	NA	NA	NA	39.3	Positive	C/S
13	Yes	2830	37.4	C/S	237.0	NA	NA	NA	NA	37.4	Positive	C/S
14	No	26 800	40.6	C/S	120.8	NA	NA	NA	NA	40.6	Positive	C/S
15	No	206	40.1	C/S	16.7	NA	NA	NA	NA	40.1	Positive	C/S

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

were then resuspended in RPMI-1640 culture medium supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies; hereafter referred to as ''supplemented RPMI''). Neutrophils were incubated in 24-well culture plates (Corning Life Sciences, Durham, North Carolina) containing 12-mm cover slips (Fisher Scientific, Waltham, Massachusetts) at a concentration of 2×10^5 cells/0.5 mL and incubated for 1 hour at 37°C with 5% CO_2. Following incubation, adherent neutrophils were stimulated using 100 nM of PMA (Sigma-Aldrich) for 2 hours at 37°C with 5% CO_2. Next, paraformaldehyde (PFA; Electron Microscopy Science, Hatfield, Pennsylvania) was added to each culture plate well at a final concentration of 4% for 2 hours. The cover slips were then carefully removed from the culture plate well, rinsed with 1X PBS, and mounted onto Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, Wilmington, Delaware) using ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Images were acquired using an Olympus BX60 fluorescence microscope (Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP71 camera and DP Controller Software (Olympus Corporation).

Amniotic fluid samples were passed through a sterile 15-μm filter (Cat# 43-50015-03; pluriSelect Life Science, Leipzig, Germany) and centrifuged at 200*g* for 5 minutes at room temperature (n = 15). Amniotic fluid cells were then resuspended in supplemented RPMI at a concentration of 2.5×10^5 cells/0.5 mL and placed in 24-well culture plates containing 12-mm cover slips. Next, cells were incubated for 1 hour at 37°C with 5% CO_2, stimulated with 100 nM of PMA for 2 hours, and fixed with 4% PFA for 2 hours. Finally, cover slips were carefully removed, rinsed with 1X PBS, and mounted onto Fisherbrand Superfrost Plus microscope slides using ProLong Diamond Antifade Mountant with DAPI. Images were acquired using an Olympus BX60 fluorescence microscope equipped with an Olympus DP71 camera and DP Controller Software.

Detection of Bacteria in Amniotic Fluid Samples

Staining of bacteria in amniotic fluid samples (n = 15) was performed as previously described,[118] using the LIVE/DEAD BacLight Bacterial Viability Kit (Cat# L7007; Life Technologies) in a sterile biosafety cabinet. Briefly, 100 μL of amniotic fluid were mixed with 900 μL of sterile 1X PBS. 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 minutes at room temperature in the dark. Next, the cells were centrifuged at 10 000*g* for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended in 5 μL of 1X PBS, and a slide smear was prepared and air-dried. Finally, the slide was gently rinsed with 1X PBS and mounted with ProLong Diamond Antifade Mountant with DAPI. Images were acquired using an Olympus BX 60 fluorescence microscope with an Olympus DP71 camera and DP Controller Software.

Scanning Electron Microscopy

Neutrophils were isolated from maternal blood and amniotic fluid samples and then cultured on cover slips, as described above (n = 3 each). After PMA stimulation, the culture medium was aspirated and electron microscopy fixative (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4; Cat# 16537-05, Electron Microscopy Science) was carefully added to the culture plate wells. Following fixation for 2 hours at 4°C, the cover slips were gently washed with 1X electron microscopy wash buffer (Sorensen's phosphate buffer 0.2 M, pH 7.4; Cat# 11601-10, Electron Microscopy Science). The cover slips were then stored in 1X electron microscopy wash buffer and transported to the Microscopy & Image Analysis Laboratory at the University of Michigan (Ann Arbor, Michigan) (https://medicine.umich.edu/medschool/research/office-research/biomedical-research-core-facilities/microscopy-image-analysis). Images were obtained using an AMRAY 1910 Field Emission Scanning Electron Microscope (SEMTechSolutions, North Billerica, Massachusetts).

Confocal Microscopy

Neutrophils from amniotic fluid samples were cultured on cover slips, as described above (n = 3). After PFA fixation, cells were washed with 1X PBS, permeabilized with 1X PBS containing 0.25% Triton X-100 (Cat# H5141; Promega, Madison, Wisconsin) for 2 minutes, and then washed with 1X PBS again. Nonspecific antibody interactions were blocked by treating the cover slips with Dako Protein Block Serum-Free Solution (catalog number X0909; DakoCytomation, Carpinteria, California) for 30 minutes at room temperature. The cover slips were incubated for 1 hour at room temperature with a mouse anti-human neutrophil elastase (Cat# M0752; clone NP57, Dako, Denmark) and a rabbit anti-human H3 antibody (Cat# ab5103; Abcam, Cambridge, Massachusetts), and then washed with 1X PBS. Next, a second blocking step was performed by adding 10% goat serum (KPL, Gaithersburg, Maryland) for 10 minutes. The cover slips were incubated with a secondary goat anti-mouse IgG–Alexa Fluor 488 antibody (Cat# A11029; Life Technologies) and a goat anti-rabbit IgG–Alexa Fluor 594 antibody (Cat#A11072; Life Technologies) for 1 hour at room temperature in the dark. Finally, the cover slips were washed with 1X PBS and mounted onto Fisherbrand Superfrost Plus microscope slides using ProLong Diamond Antifade Mountant with DAPI. Slides 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 (Detroit, Michigan) (http://micr.med.wayne.edu/). Confocal Z stacks were acquired using a Plan-Apochromat 100×/1.40 Oil DIC lens with 1.5× digital zoom. Immunofluorescence signals for Alexa Fluor 594 and Alexa Fluor 488 were excited with an In Tune White Light laser tuned to 595 nm and a 488-nm line Multiline Argon

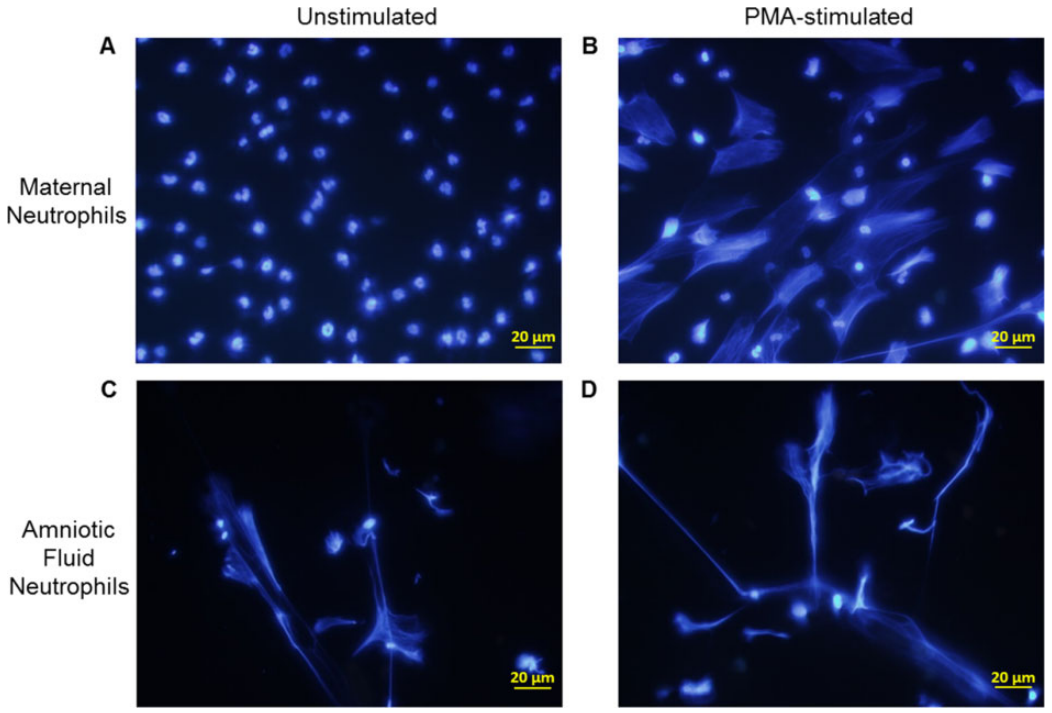

Figure 1. DAPI staining of NETs in maternal peripheral blood and the amniotic fluid. Representative fluorescence microscopy images of (A) unstimulated maternal neutrophils, (B) PMA-stimulated maternal neutrophils, (C) unstimulated amniotic fluid neutrophils, and (D) PMA-stimulated amniotic fluid neutrophils. DNA is blue (DAPI). Magnification 400×. DAPI indicates 4′,6-diamidino-2-phenylindole; NET, neutrophil extracellular trap; PMA, phorbol-12-myristate-13-acetate. (The color version of this figure is available online.)

laser, respectively. The DAPI signal was excited with a 405-nm diode laser.

Determination of NET Formation by Maternal and Neonatal Neutrophils Incubated with the Amniotic Fluid of Women without Intra-Amniotic Infection

Maternal or neonatal neutrophils were isolated from peripheral or cord blood, respectively, using density gradients and cultured on cover slips, as described above. Five hundred microliters of amniotic fluid from 4 singleton term pregnancies without labor or infection/inflammation (Gram stain negative, negative cultures, WBC count = 0, and IL-6 < 2.6 ng/mL) were added to the cultured neutrophils and incubated for 2 hours at 37°C with 5% CO_2. Positive controls included maternal or neonatal neutrophils stimulated with PMA, as described above. Next, PFA was added to each culture plate well at a final concentration of 4% for 2 hours. The cover slips were then carefully removed from the culture plate well, rinsed with 1X PBS, and mounted onto Fisherbrand Superfrost Plus microscope slides using ProLong Diamond Antifade Mountant with DAPI. Images were acquired using an Olympus BX60 fluorescence microscope equipped with an Olympus DP71 camera and DP Controller Software.

Results

Characteristics of the Study Population

A total of 15 amniotic fluid samples from women who underwent transabdominal amniocentesis before delivery or during cesarean section were included in this study. Demographic and clinical characteristics of the study population are displayed in Table 1. All of the patients were diagnosed with intra-amniotic inflammation as they had elevated concentrations of IL-6 in the amniotic fluid (≥ 2.6 ng/mL[92,93]; Table 2). Nine patients underwent spontaneous labor at term (Table 1), of which 7 were diagnosed with clinical chorioamnionitis (Table 2). Five patients underwent spontaneous preterm labor and birth, and 1 patient delivered preterm in the absence of labor (Table 1). Five of the 6 patients who delivered preterm were diagnosed with clinical chorioamnionitis (Table 2). All of the patients who underwent amniocentesis for clinical purposes were diagnosed with intra-amniotic infection as they had positive cultures (ie, MIAC) and elevated concentrations of IL-6 in the amniotic fluid (≥ 2.6 ng/mL[3-6,10,92-102]; Table 2). All of these patients also had elevated WBC counts (≥ 50 cells/mm^3)[19] in the amniotic fluid (Table 2). Most of the patients (10/11) had low glucose concentrations (< 14 mg/dL)[115] in the amniotic fluid (Table 2). The most common microorganisms were *Ureaplasma* subspecies followed by *Streptococcus* subspecies (Table 2).

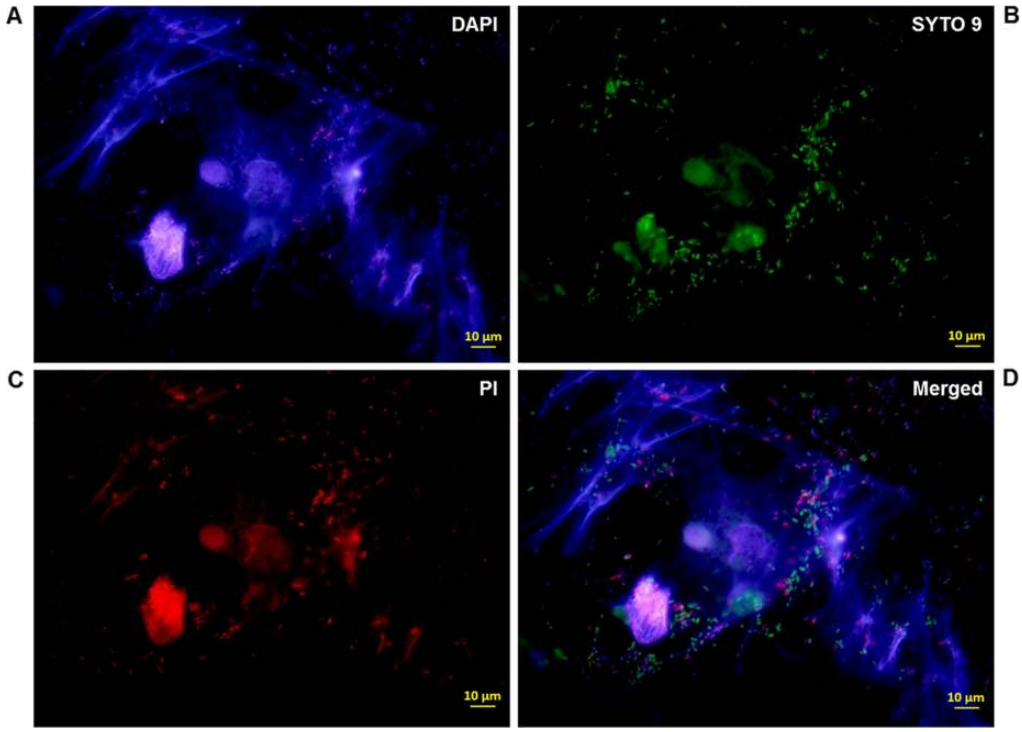

Figure 2. Bacterial Live/Dead staining and DAPI staining of an amniotic fluid sample showing bacteria trapped by NETs. Separated layers show (A) DAPI staining, (B) SYTO 9 staining, (C) propidium iodide (PI) staining, and (D) a merged image. Live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red. DNA is blue (DAPI). Magnification 1000×. DAPI indicates 4′,6-diamidino-2-phenylindole; NET, neutrophil extracellular trap. (The color version of this figure is available online.)

The First Observation of NETs in the Amniotic Cavity of Women with Intra-Amniotic Inflammation

First, we investigated whether NETs were detectable in amniotic fluid samples from women with intra-amniotic inflammation. As a positive control, we stimulated neutrophils from maternal blood with PMA and observed the appearance of NETs using DAPI staining, which detects DNA.[119,120] Figure 1A and 1B demonstrate that incubation of maternal neutrophils with PMA induces the formation of NETs.[78,117] However, amniotic fluid neutrophils formed NETs in the absence of PMA stimulation (Figure 1C). When amniotic fluid neutrophils were stimulated with PMA, no evident increase in NET formation was observed.

Detection of NETs and Bacteria in the Amniotic Fluid

In order to investigate whether the presence of bacteria in the amniotic fluid was associated with the production of NETs, we utilized bacterial Live/Dead staining combined with DAPI staining.[118] All of the amniotic fluid samples had detectable live and dead bacteria (Table 2). It is worth mentioning that the bacterial Live/Dead staining detected *Ureaplasma* subspecies and Mycoplasma subspecies in sample #8 (Table 2); yet, further validation of this assay is required to identify bacteria without a cell wall in the amniotic fluid. Figure 2 is a representative image of live and dead bacteria trapped by amniotic fluid NETs. These data demonstrate that all of the patients had intra-amniotic infection, and more importantly, that NETs are associated with MIAC.

Visualization of NETs by Scanning Electron Microscopy

We further characterized the appearance of NETs in the amniotic fluid using scanning electron microscopy. Maternal peripheral neutrophils without PMA stimulation maintained a classic round morphology typical of quiescent cells[80,121-123] (Figure 3A and B). Maternal peripheral neutrophils stimulated with PMA appeared amorphous and distended, with filamentous projections, possibly DNA, being released (Figure 3C and D).[80,123] The morphology of amniotic fluid neutrophils resembled PMA-stimulated maternal neutrophils (Figure 3E and F). Yet, the amniotic fluid neutrophils appeared flatter and more distended without the filamentous projections observed in PMA-stimulated maternal neutrophils (Figure 3E vs. C).

Visualization of Elastase and Histone H3 in Amniotic Fluid NETs

Next, we used confocal microscopy to confirm that the structures observed in the amniotic fluid were indeed NETs. We then performed elastase and histone H3 staining of two known components of NETs,[78,117,124-127] in combination with DAPI staining. In the amniotic fluid, there were neutrophils in a

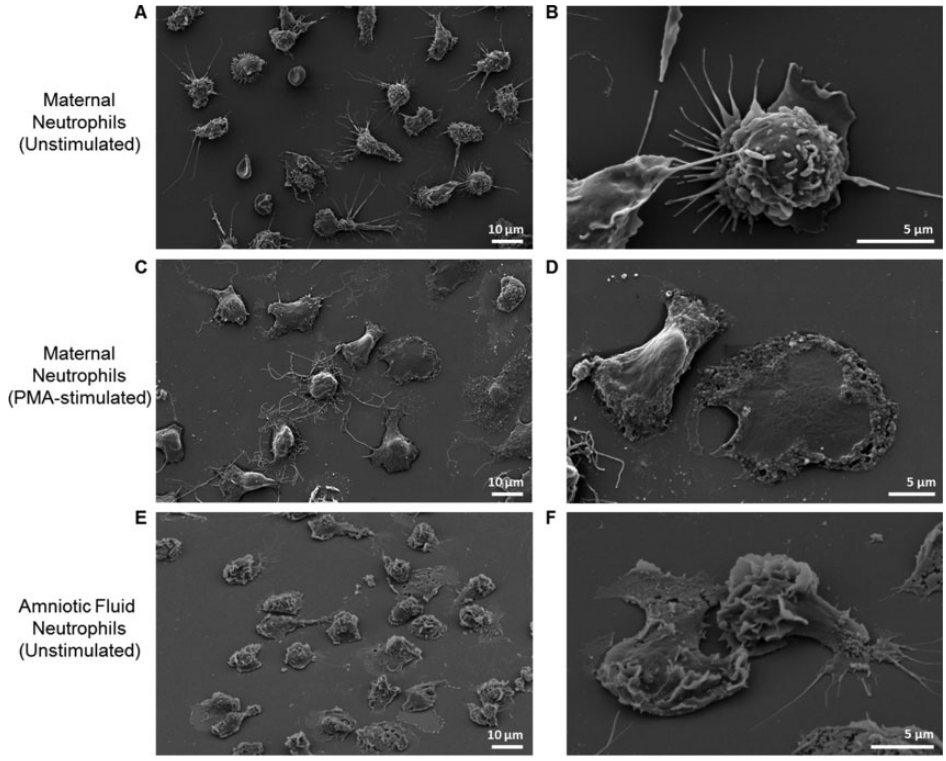

Figure 3. Scanning electron microscopy of neutrophils from maternal peripheral blood and amniotic fluid samples. Unstimulated maternal neutrophils were captured at magnifications (A) 1000× and (B) 5000×, PMA-stimulated maternal neutrophils were captured at magnifications (C) 1000× and (D) 3000×, and unstimulated neutrophils from amniotic fluid were captured at magnifications (E) 1000× and (F) 4000×. PMA indicates phorbol-12-myristate-13-acetate.

resting stage (Figure 4; white arrow) and at different stages of NET formation (Figures 4 and 5). In Figure 4, the yellow arrow represents an early stage of NET formation as the neutrophil shows extranuclear histone H3 and intracellular DNA and elastase. The red arrow in Figure 4 represents an intermediate stage of NET formation as elastase is bursting out of the cell; yet, DNA and histones are still contained. In Figure 5, the final stage of NET formation is observed as histone H3, elastase, and DNA burst from an amniotic fluid neutrophil. Altogether, these data demonstrate that different stages of NET formation are present in the amniotic cavity of women with intra-amniotic infection.

Maternal or Neonatal Neutrophils Do Not Form NETs in the Amniotic Fluid of Women without Intra-Amniotic Infection

In order to prove that amniotic fluid neutrophils do not form NETs in the absence of bacteria, maternal or neonatal neutrophils were added to amniotic fluid samples from women who delivered at term without intra-amniotic infection. PMA induced in vitro NET formation in maternal and neonatal neutrophils (Figure 6: A vs. B and D vs. E). Neonatal neutrophils formed PMA-induced NETs at a lesser extent than maternal neutrophils, as previously demonstrated (Figure 6E vs. B).[128,129] However, maternal and neonatal neutrophils incubated with amniotic fluid samples from women without intra-amniotic infection did not form NETs in vitro (Figure 6C and F). These results show that maternal and neonatal neutrophils do not form NETs in the amniotic fluid in the absence of bacteria or inflammation.

Discussion

Amniotic fluid neutrophils are considered to be of fetal origin,[49,50] and their number is a useful marker for intra-amniotic inflammation.[19] Yet, amniotic fluid neutrophils have been observed in patients with a severe maternal inflammatory response without a fetal inflammatory response (ie, funisitis and chorionic vasculitis), suggesting that, in some cases, amniotic fluid neutrophils are of maternal origin or a mixture of both fetal and maternal neutrophils. Previously, we provided evidence that amniotic fluid neutrophils are a source of antimicrobial peptides[130] and cytokines[51] in humans. However, their precise role in immune host defense needs to be further elucidated. Herein, we demonstrated for the first time that amniotic fluid neutrophils form NETs in patients with intra-amniotic infection. As NET formation represents a mechanism of innate immune defense against pathogens,[83,131] we propose that amniotic fluid neutrophils form extracellular traps to kill microbes invading the amniotic cavity.

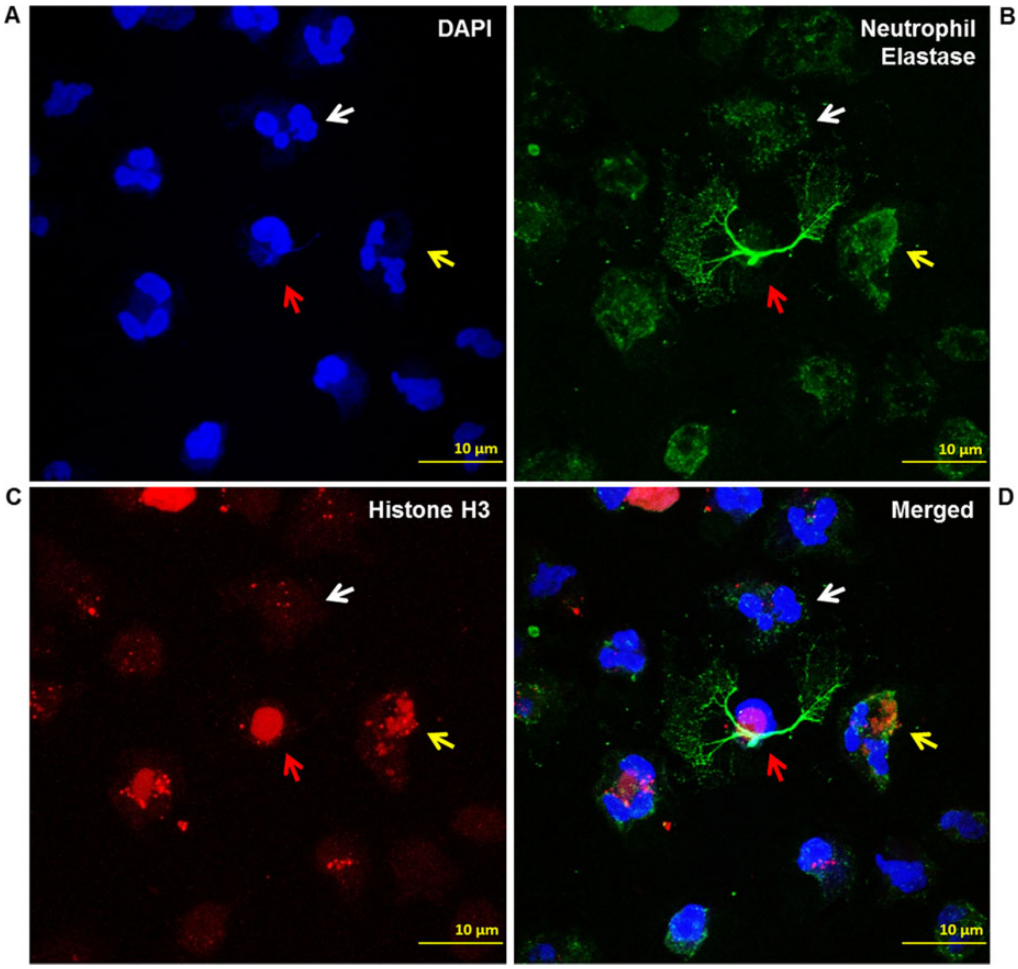

Figure 4. Confocal microscopy showing different stages of NET formation in amniotic fluid samples. Separated layers show (A) DAPI staining in blue, (B) neutrophil elastase staining in green, (C) histone H3 staining in red, and (D) a merged image. White arrows point to a neutrophil that is in a resting stage, yellow arrows point to a neutrophil in an early stage of NET formation, and red arrows point to a neutrophil in an intermediate stage of NET formation. Magnification 630×. DAPI indicates 4′,6-diamidino-2-phenylindole; NET, neutrophil extracellular trap. (The color version of this figure is available online.)

NET formation is induced in vivo in response to *Staphylococcus aureus*,[79] *S pyogenes*,[132] *Escherichia coli*,[133] *Candida albicans*,[134] *Aspergillus nidulans*,[135] *Leishmania amazonensis,*[136] and HIV-1.[137] Bacteria lacking a cell wall (eg, Mycoplasma subspecies) can also induce NET formation.[138] Furthermore, NETs are abundant in placental villi from preeclamptic women.[139] In the present study, we demonstrated that amniotic fluid neutrophils undergo NET formation in intra-amniotic infections mostly due to *Ureaplasma* subspecies and/or *Streptococcus* subspecies (mostly *Streptococcus agalactiae* or group B streptococcus [GBS]). The mechanism whereby *Ureaplasma* subspecies induces NET formation could involve toll-like receptor (TLR) signaling as the intra-uterine inoculation of *Ureaplasma parvum* increases the expression of TLRs 1, 2, and 6 in placental tissues.[140]

GBS induces NET formation in the genital tract (ie, cervicovaginal swabs) when inoculated vaginally.[141] Incubation with this Gram-positive bacterium also induces NET formation in peripheral blood neutrophils in vitro.[141] Importantly, GBS can evade NET formation by degrading the DNA matrix comprising the extracellular trap.[142]

We noted that some of the bacteria trapped by amniotic fluid NETs were alive at the time of staining. This finding could represent that (1) the process of NET formation was in its early stages, (2) amniotic fluid NETs could not kill bacteria efficiently, or (3) some bacteria, such as GBS, can degrade the extracellular trap. Further studies are required to investigate the mechanisms whereby *Ureaplasma* subspecies, *Streptococcus* subspecies, and other bacteria involved in intra-amniotic infection induce NET formation.

In the current study, we also demonstrated that neither maternal nor neonatal neutrophils form NETs in the amniotic fluid of women without intra-amniotic infection. This finding suggests that amniotic fluid neutrophils form NETs when bacteria invade the amniotic cavity. Yet, there is a possibility that NETs are formed in the setting of sterile intra-amniotic inflammation as alarmins (eg, high-mobility group box 1,[143] monosodium urate crystals,[144] and heme[145]) and cytokines

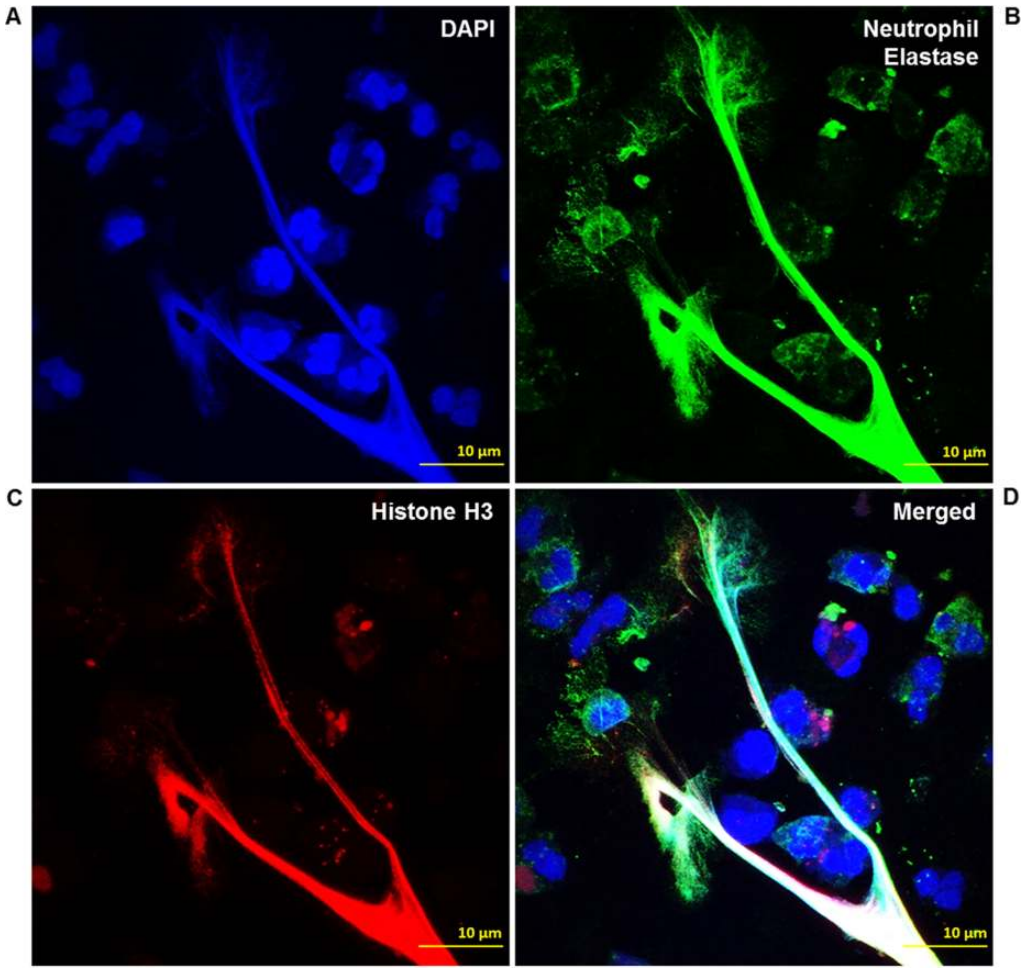

Figure 5. Confocal microscopy showing the late stage of NET formation in amniotic fluid samples. Separated layers show (A) DAPI staining in blue, (B) neutrophil elastase staining in green, (C) histone H3 staining in red, and (D) a merged image. Magnification 630×. DAPI indicates 4′,6-diamidino-2-phenylindole; NET, neutrophil extracellular trap. (The color version of this figure is available online.)

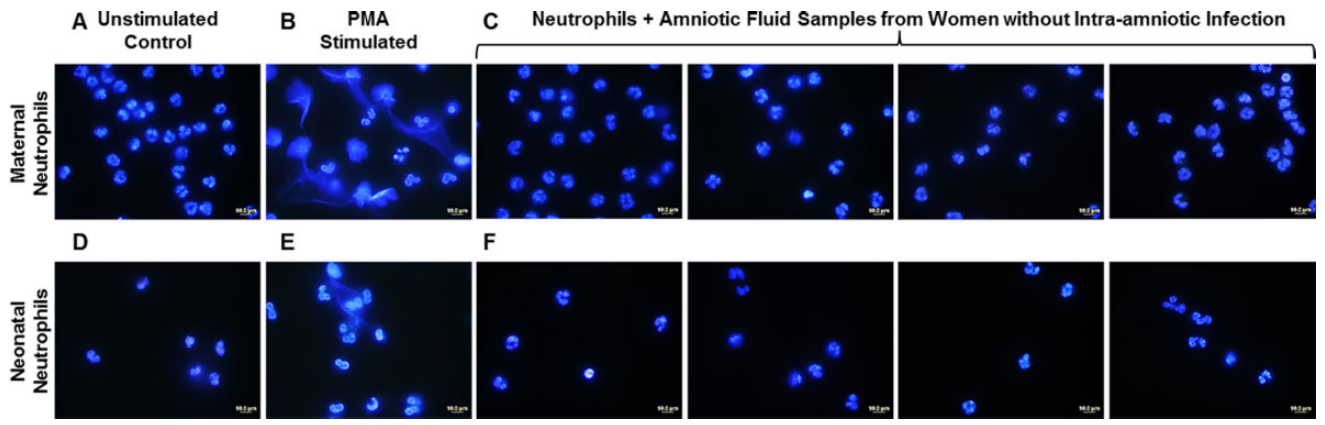

Figure 6. DAPI staining of maternal and neonatal neutrophils in the amniotic fluid of women without intra-amniotic infection. Representative fluorescence microscopy images of (A) unstimulated maternal neutrophils, (B) PMA-stimulated maternal neutrophils, (C) maternal neutrophils incubated with 4 different amniotic fluid samples from women without intra-amniotic infection, (D) unstimulated neonatal neutrophils, (E) PMA-stimulated neonatal neutrophils, and (F) neonatal neutrophils incubated with 4 different amniotic fluid samples from women without intra-amniotic infection. DNA is blue (DAPI). Magnification 1000×. DAPI indicates 4′,6-diamidino-2-phenylindole; PMA, phorbol-12-myristate-13-acetate. (The color version of this figure is available online.)

(eg, IL-1β[144] and IL-8[78]) induce in vitro NET formation in the absence of bacteria.

It is worth mentioning that neonatal neutrophils formed fewer NETs than maternal neutrophils upon PMA stimulation. This impairment was recently attributed to a neonatal NET-inhibitory factor present in cord blood plasma, which blunts in vitro and in vivo NET formation.[145]

In summary, we report that amniotic fluid neutrophils undergo NET formation in women with intra-amniotic infection. This finding provides a new immune defense mechanism whereby amniotic fluid neutrophils can kill microbes invading the amniotic cavity.

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Declaration of Conflicting Interests

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