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Clinical Chorioamnionitis at Term VII: The Amniotic Fluid Cellular Immune Response

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Abstract

Objective—1) To characterize the cellular composition of the amniotic fluid of patients diagnosed with clinical chorioamnionitis at term, as a function of the presence or absence of microorganisms determined by cultivation techniques, and 2) to characterize the cytokine production by white blood cells present in the amniotic fluid using flow cytometry-based techniques.

Material and Methods—Amniotic fluid samples from 20 women with the diagnosis of clinical chorioamnionitis at term were analyzed using cultivation techniques (for aerobic and anaerobic bacteria, as well as genital Mycoplasmas). Amniotic fluid IL-6 concentrations were determined by an enzyme-linked immunosorbent assay. Amniotic fluid leukocytes were visualized by using hematoxylin and eosin staining and immunofluorescence. Immunophenotyping of surface markers and cytokines was performed in amniotic fluid leukocytes using flow cytometry.

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Disclosure/Conflict of Interest

The authors disclose no conflicts of interest.

Results—1) Neutrophils (CD45+ CD15+ cells) were the most common leukocyte subset found in the amniotic fluid, followed by monocytes (CD45+ CD14+ cells); other white blood cells (such as lymphocytes and natural killer cells) were scarce in the amniotic fluid; 2) the absolute counts of neutrophils and monocytes were significantly higher in patients with microorganisms found in the amniotic fluid than in those without detectable microorganisms, using cultivation techniques; 3) there was a significant correlation between the absolute counts of neutrophils and monocytes determined by flow cytometry (Spearman's correlation = 0.97; $p < 0.001$); 4) there was a significant correlation between the absolute white blood cell count determined with a hemocytometer chamber and flow cytometric analysis (Spearman's correlation = 0.88; $p<0.001$); and 5) the profile of cytokine expression differed between monocytes and neutrophils; while neutrophils predominantly produced TNF-α and MIP-1β, monocytes expressed higher levels of IL-1β and IL-1α.

Conclusion—Flow cytometry analysis of the amniotic fluid of patients with intra-amniotic infection and clinical chorioamnionitis at term demonstrated that neutrophils and monocytes are the most common cells participating in the inflammatory process. We have characterized for the first time the differential cytokine expression by these cells in this important complication of pregnancy.

Keywords

cytokine; flow cytometry; interleukin 1-alpha (IL-1α); interleukin 1-beta (IL-1β); interleukin-6 (IL-6); leukocytes; microbial invasion of the amniotic cavity (MIAC); monocytes; neutrophils; parturition

Introduction

Clinical chorioamnionitis at term is the most common diagnosis related to infection made in labor and delivery units world-wide [1–8]. This term refers to a clinical syndrome traditionally attributed to microbial invasion of the amniotic cavity frequently caused by genital Mycoplasmas, *Gardnerella vaginalis*, and other commensal organisms observed in the lower genital tract [9, 10]. Microorganisms and their products can elicit a local inflammatory response in the amniotic cavity [11–17], termed intra-amniotic inflammation [18–27], which may extend to two hosts – the fetus [28–31] and mother [32]. Clinical chorioamnionitis represents evidence of a systemic maternal inflammatory response [32] but not necessarily a fetal inflammatory response [31].

The intra-amniotic inflammatory response is characterized by an increased amniotic fluid white blood cell count [33–37] and increased concentrations of inflammatory mediators, such as cytokines [27, 34–36, 38–75] and prostaglandins [76–85]. However, there is a paucity of information about the cellular composition of the intra-amniotic inflammatory response in clinical chorioamnionitis, and most studies have reported changes in the total white blood cell count of the amniotic fluid while using a hemocytometer chamber (e.g. a Neubauer chamber).

Flow cytometry has emerged as a state-of-the-art procedure to assess the phenotypic characteristics of cells participating in inflammatory responses [86–89]. This technique

characterizes their functional state (i.e. assesses reactive oxygen species and cytokine production) [90–94], determines viability [95–97], and evaluates other important biological characteristics [98–103].

The purpose of this study was to characterize, by flow cytometry, the cellular composition of the amniotic fluid from patients diagnosed with clinical chorioamnionitis at term, as a function of the presence or absence of microorganisms determined by conventional cultivation techniques. Moreover, this study was designed to characterize cytokine production by white blood cells present in the amniotic fluid by using flow cytometry-based techniques.

Material and methods

Study population

This was a cross-sectional study of patients diagnosed with clinical chorioamnionitis at term who underwent transabdominal amniocentesis to identify microorganisms in the amniotic cavity. Patients were enrolled at Hutzel Women's Hospital of the Detroit Medical Center (November 2013 – July 2015). Inclusion criteria were as follows: 1) singleton gestation; 2) gestational age ≥37 weeks; and 3) sufficient amniotic fluid obtained by transabdominal amniocentesis for immunophenotyping using flow cytometry. In all cases, an amniocentesis was performed for clinical indications.

Maternal and neonatal data were obtained from retrospective clinical chart reviews; the information included: 1) the use of epidural analgesics and intrapartum antibiotics, 2) the membrane status (intact or ruptured) at the time of amniocentesis, and 3) the mode of delivery. Patients diagnosed with clinical chorioamnionitis (see diagnostic criteria below) 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 NICHD and Wayne State University.

Clinical definitions

Gestational age was determined by the date of the last menstrual period and confirmed by an 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 (temperature >37.8°C) accompanied by two or more of the following criteria: 1) uterine tenderness; 2) malodorous vaginal discharge; 3) fetal tachycardia (heart rate >160 beats/minute); 4) maternal tachycardia (heart rate >100 beats/minute); and 5) maternal leukocytosis (leukocyte count >15,000 cells/mm^3) [2–4,6]. Spontaneous term labor was defined as the presence of regular uterine contractions with a frequency of at least one every 10 minutes and cervical change after 37 weeks of gestation.

Microbial invasion of the amniotic cavity was defined according to the results of the amniotic fluid cultures [104–108]. Intra-amniotic inflammation was diagnosed when the amniotic fluid interleukin-6 (IL-6) concentration was ≥2.6 ng/mL [51, 109]. Based on the results of the amniotic fluid cultures, patients were classified as having either a negative or a positive culture. Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes [110–113], while acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or in the Wharton's jelly, using criteria previously described [110, 113–117].

Sample collection

Amniotic fluid was retrieved by transabdominal amniocentesis, which was performed under antiseptic conditions, using a 22-gauge needle monitored by ultrasound. Each sample of amniotic fluid was transported to the clinical laboratory in a capped sterile syringe and was cultured for aerobic and anaerobic bacteria as well as for genital Mycoplasmas [10,33–37,48,118–120]. Shortly after collection, a white blood cell count was performed for each amniotic fluid sample using a hemocytometer chamber, according to methods previously described [33]. Glucose concentrations [121] were determined and Gram stains [122,123] were performed on the amniotic fluid samples.

Determination of IL-6 in amniotic fluid

Concentrations of IL-6 in the amniotic fluid were determined by 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. The IL-6 concentrations in the amniotic fluid were determined for clinical purposes. It has been previously reported that IL-6 concentrations are useful for the detection of intra-amniotic inflammation [51, 109].

Hematoxylin and eosin staining

Amniotic fluid cells were counted using an automatic cell counter (Cellometer Auto 2000; Nexcelom, Lawrence, MA). Amniotic fluid cells (5×10^4) were cytospun onto a Fisherbrand Superfrost Plus microscope slide (Thermo Fisher Scientific, Rochester, NY) using a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific) at 800 rpm for 5 minutes. The slide was then stained with hematoxylin (catalog number 88018, Thermo Scientific) for 10 seconds, washed with distilled water, immersed in 80% ethanol, and stained with eosin (catalog number 71211, Thermo Scientific) for 10 seconds. Next, each slide was dehydrated by subsequent washes with 95% ethanol, 100% ethanol, and xylene, and was mounted with xylene. Images were obtained using a Pannoramic MIDI slide scanner (PerkinElmer, Waltham, MA).

Immunofluorescence

Amniotic fluid cells (5×10^4) were cytospun onto a Fisherbrand Superfrost Plus microscope slide (Thermo Fisher Scientific) using a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher

Scientific) at 800 rpm for 5 minutes. The cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and washed with 1X phosphate-buffered saline (1X PBS; Life Technologies, Grand Island, NY). Non-specific antibody interaction was blocked using Dako Protein Block Serum-Free Solution (catalog number X0909; DakoCytomation, Carpinteria, CA) for 30 minutes at room temperature. Next, cells were incubated with mouse anti-human CD14 (clone MφP9, catalog number 347490, BD Biosciences, San Jose, CA) for 1 hour at room temperature. Cells were then washed with 1X PBS containing 0.1% Tween 20 (PBST) (Sigma-Aldrich, St. Louis, MO). After blocking for 10 minutes with 10% goat serum (KPL, Gaithersburg, MD), a secondary goat anti-mouse IgG–Alexa Fluor 594 (catalog number A11032; Life Technologies) was added and cells were incubated for 1 hour at room temperature in the dark. Following incubation, cells were washed with PBST and incubated with FITC-conjugated mouse anti-human CD15 (clone W6D3, catalog number 5623790, BD Biosciences) for 1 hour at room temperature in the dark. Finally, cells were washed with PBST and then mounted using ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Immunofluorescence was visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using an Olympus DP71 camera and DP Controller software (Olympus).

Immunophenotyping of amniotic fluid leukocytes by flow cytometry

Amniotic fluid samples (1.5 mL) were washed with 1 mL of 1X PBS and the resulting cell pellet was re-suspended in 1 mL of 1X PBS. One microliter of the Fixable Viability Stain 510 reagent (catalog number 564406; BD Biosciences) was added to the cell suspension, which was incubated for 30 minutes at 4°C in the dark. Following incubation, the cell suspension was centrifuged at 285 × g and the cell pellet was washed with 1 mL of FACS buffer [BSA 0.1% (Sigma-Aldrich)], sodium azide 0.05% [Fisher Scientific Bioreagents, Fair Lawn, NJ], and 1X PBS)]. The cell pellet was then incubated with 10 μL of human FcR blocking reagent (Miltenyi Biotec, San Diego, CA) in 40 μL of stain buffer (catalog number 554656; BD Biosciences) for 10 minutes at 4°C. Next, the cells were incubated with the following extracellular fluorochrome-conjugated anti-human antibodies (BD Biosciences) for 30 minutes at 4°C in the dark: CD45-Alexa Fluor 700 (clone HI30), CD15-Brilliant Violet 650 (clone HI98), CD14-Brilliant Ultraviolet 395 (clone MφP9), CD19-PE-Cy5 (clone HIB19), CD3-APC-Cy7 (clone SK7), and CD56-Brilliant Violet 711 (clone NCAM18.2). After extracellular staining, the cells were washed with 1X PBS to remove excess antibody, re-suspended in 0.5 mL of stain buffer, and acquired using the BD LSRFortessa flow cytometer (BD Biosciences) and BD FACSDiva 6.0 software (BD Biosciences). The analysis and figures were performed using the FlowJo software version 10 (FlowJo, Ashland, OR). Neutrophils and monocytes were defined as CD45+CD15+ cells and CD45+CD14+ cells, respectively. The total number of neutrophils and monocytes was determined using CountBright absolute counting beads (Molecular Probes, Eugene, OR).

Cytokine expression assessed by flow cytometry

Cytokine expression was assessed by intracellular staining. Following extracellular staining, the cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences). Next, the cells were washed with 1X BD Perm/Wash Buffer (BD Biosciences), re-suspended in 50 μL of the same buffer, and stained with

the following intracellular antibodies (BD Biosciences and Biolegend, San Diego, CA) for 30 minutes at 4°C in the dark: macrophage inflammatory protein (MIP)-1α-PE (clone 11A3), MIP-1β-PerCP-Cy5.5 (clone D21-1351), IL-1α-FITC (clone A55), IL-1β-Alexa Fluor 647 (clone JK1B-1), IL-8-Brilliant Violet 421 (clone G265-8), and TNF-α-Brilliant Violet 605 (clone Mab11). Isotype controls were also prepared. Finally, the stained cells were washed with 1X BD Perm/Wash Buffer, re-suspended in 0.5 mL of stain buffer, and acquired using the BD LSR II and LSRFortessa flow cytometers and BD FACSDiva 6.0 software. The analysis was performed using FlowJo software version 10. The mean fluorescence intensity (MFI) of every cytokine was calculated as [MFI (anti-cytokine mAb) – MFI (isotype control mAb)].

Statistical analysis

Statistical analysis was performed using the R package [124]. Normality of the data was tested using the Wilk-Shapiro test. Mann-Whitney U-tests were used to compare the medians among the groups. Comparisons of the proportions were made using Fisher's exact tests. Spearman's correlation was used to examine the relationship between continuous variables. A p-value <0.05 was used to determine statistical significance.

Results

Characteristics of the study population

A total of 20 patients diagnosed with clinical chorioamnionitis between 37 and 42 weeks of gestation were included in this study. Demographic and clinical characteristics of the study population are displayed in Table 1. Seven patients had amniotic fluid cultures negative for bacteria, and 13 patients had positive cultures. There were no significant differences in the median maternal age, frequency of nulliparity, pre-pregnancy body mass index, gestational age at amniocentesis and at delivery, frequency of vaginal delivery, and birthweight among the groups.

Patients with amniotic fluid cultures positive for microorganisms had a higher frequency of ruptured membranes than those with negative amniotic fluid cultures [92.3% (12/13) vs. 28.6% (2/7); p=0.008]. Patients with positive amniotic fluid cultures had significantly higher median amniotic fluid total white blood cell counts and IL-6 concentrations, as well as lower amniotic fluid glucose concentrations, than women with negative amniotic fluid cultures.

Funisitis was more common in women with positive amniotic fluid cultures than in those with negative amniotic fluid cultures [92.3% (12/13) vs. 28.6% (2/7), p=0.008].

Table 2 describes microorganisms in the amniotic fluid inflammatory response as well as white blood cell counts determined by flow cytometry in patients with positive amniotic fluid cultures.

Neutrophil and monocyte counts are higher in patients with positive amniotic fluid cultures than in those with negative amniotic fluid cultures

Figure 1 represents the gating strategy used to identify leukocytes in the amniotic fluid samples. Briefly, total leukocytes were identified using CD45 within the viability gate.

Leukocytes expressing CD15 were considered to be neutrophils and those expressing CD14 were considered to be monocytes. Flow cytometry demonstrated that neutrophils and monocytes were the most abundant leukocyte subsets observed in the amniotic fluid of patients with clinical chorioamnionitis at term and positive amniotic fluid cultures (Figure 1). Lymphocytes (CD45+CD3+ and CD45+CD19+ cells) and natural killer cells (CD45+CD56+ cells) were scarce in the amniotic fluid samples. Hematoxylin and eosin staining of the amniotic fluid exudate showed the typical morphology of neutrophils (N; green arrow) and monocytes (M; red arrow) (Figure 2A). Immunofluorescence of these amniotic fluid cells also revealed that neutrophils (CD15+ cells are green) and monocytes (CD14+ cells are red) are abundant in the amniotic fluid of patients with clinical chorioamnionitis at term (Figure 2B).

The median neutrophil count was higher in patients with amniotic fluid cultures positive for microorganisms than in those with negative amniotic fluid cultures [193.1 cells/mm^3 (18.4–543.8) vs. 0.4 cells/mm^3 (0.1–8.2); p=0.01] (Figure 3). Similarly, the median monocyte count was significantly higher in those with positive (vs. negative) amniotic fluid culture for microorganisms [6.5 cells/mm^3 (0.3–10.9) vs. 0 cells/mm^3 (0.0–0.4); p=0.01] (Figure 3). There was a significant positive correlation between the amniotic fluid total white blood cell counts determined by flow cytometry and a hemocytometer chamber (Spearman's correlation = 0.88; $p < 0.001$; Table 3, Figure 4). There was a significant positive correlation between the amniotic fluid neutrophil and monocyte counts determined by flow cytometry (Spearman's correlation = 0.97; $p < 0.001$; Table 3). There were significant positive correlations between IL-6 concentrations and the amniotic fluid neutrophil (Spearman's correlation = 0.59; p = 0.007) and monocyte (Spearman's correlation = 0.61; p = 0.004) counts determined by flow cytometry (Table 3). The organisms involved as well as the number of neutrophils and monocytes are described in Table 2. The most common microorganism was *Ureaplasma* species followed by *Streptococcus agalactiae* and *Gardnerella vaginalis*.

Cytokine expression by neutrophils and monocytes

Cytokine expression by the amniotic fluid white blood cells was studied in patients with positive amniotic fluid cultures. Patients with clinical chorioamnionitis but negative amniotic fluid cultures did not have enough cells in 1.5mL to study cytokine expression. Neutrophils had a higher median mean fluorescence intensity (MFI) for TNF-α and MIP-1β than monocytes (Figures 5A and 5B). In contrast, monocytes had a higher MFI for IL-1α and IL-1β than neutrophils (Figures 5C and 5D). The median MFI for IL-8 and MIP-1α was not significantly different between neutrophils and monocytes (Figures 5E and 5F).

Discussion

Principal findings of the study

The study herein is a novel report about the cytometric characteristics of the amniotic fluid from patients diagnosed with clinical chorioamnionitis at term. The principal findings of the study are as follows: 1) neutrophils (CD45+CD15+ cells) were the most common leukocyte subset found in the amniotic fluid, followed by monocytes (CD45+CD14+ cells); other

white blood cells (such as lymphocytes and natural killer cells) were scarce in the amniotic fluid; 2) the absolute counts of neutrophils and monocytes were significantly higher in patients with detectable microorganisms in the amniotic fluid than in those without detectable microorganisms, using cultivation techniques; 3) there was a significant correlation between the absolute counts of neutrophils and monocytes, determined by flow cytometry ($r = 0.97$; $p < 0.001$), and also between the absolute white blood cell count determined with a hemocytometer chamber and the counts derived from flow cytometric analysis ($r = 0.88$; $p < 0.001$); and 4) the profile of cytokine expression was different for monocytes and neutrophils; while neutrophils predominantly expressed TNF-α and MIP-1β, monocytes expressed higher levels of IL-1α and IL-1β. Collectively, these findings suggest that the inflammatory exudate in intra-amniotic inflammation is complex and that neutrophils and monocytes play different roles in clinical chorioamnionitis at term.

Flow cytometric analysis of amniotic fluid

The standard method to enumerate white blood cells in the amniotic fluid utilizes a hemocytometer chamber (e.g. a Neubauer chamber) [33–37, 125] and a cytospin to perform a differential count [33]. Flow cytometry allows the identification of cell populations using specific surface markers and also the examination of functional properties such as cytokine production [86–93,126]. The present study was undertaken to gain insights into the nature of the inflammatory response in clinical chorioamnionitis at term and also to study, for the first time, the cytokine profile of leukocytes involved in intra-amniotic infection. The observation that neutrophils are the cells most commonly found in intra-amniotic infection is consistent with previous studies [33]. The fact that monocytes are the second most common cell is also consistent with prior observations [33].

Amniotic fluid neutrophils

Neutrophils are the most abundant leukocytes in the circulatory system and form the first line of defense against invading pathogens; they possess an arsenal of weapons suited for the elimination of microbes [127]. These innate immune cells are first and foremost phagocytes, capable of enveloping and digesting microbes through the generation of reactive oxygen species (ROS) and NADPH-derived oxidants [128]. Neutrophils are granulocytic, carrying granules filled with a variety of enzymes that can be injected into the phagosome or released externally [127, 128]. Along with degranulation, neutrophils release a variety of antimicrobial proteins (such as defensins) and reactive nitrogen intermediates [128]. Recently, a new mechanism was described whereby neutrophils undergo a specialized cell death, releasing web-like structures composed of DNA, histones, and antimicrobial products such as neutrophil elastase [129]. Termed “neutrophil extracellular traps,” or NETs, this phenomenon represents the final containment effort of a neutrophil to combat microbial invasion [129]. Detection of invading microbes is largely dependent on the expression of specialized surface receptors and ligands. The carbohydrate epitope 3-fucosyl-N-acetyl-lactosamine (CD15), a classical marker for neutrophil identification, is highly expressed on activated neutrophils [130]. Neutrophils are constantly circulating and require a finely tuned homing and adhesion system in order to reach sites of infection. Expression of CXCR-1 and CXCR-2 allows neutrophils to detect the potent neutrophil-specific chemokine, IL-8 [131], while L-selectin [132, 133] and platelet endothelial cell adhesion molecule-1 [134] enable

neutrophil adhesion and transmigration [128]. Along with antimicrobial mechanisms, neutrophils release cytokines in order to signal local and immune cells. Neutrophils primarily secrete pro-inflammatory cytokines such as TNF-α, IL-12, IL-1α and IL-1β [135]. As first responders, neutrophils release multiple chemokines (IL-8, MIP-1α and MIP-1β) to attract other immune cells as well as growth factors such as granulocyte-colony stimulating factor, vascular endothelial growth factor, and hepatocyte growth factor [135].

Neutrophils are the most abundant leukocytes found in the amniotic fluid in cases with intra-amniotic infection/inflammation [33]. Their fetal origin was demonstrated when X and Y chromosomes were detected by fluorescence *in situ* hybridization in the amniotic fluid leukocytes from four women with intra-amniotic infection who delivered premature male infants [136]. Since the neutrophils observed in the chorioamniotic membranes are of maternal origin and the chorionic plate contains a mix of maternal and fetal neutrophils, it has been suggested that the fetal vasculature of the chorionic plate is the main source of amniotic fluid neutrophils [137]. Yet, a more comprehensive study that would include a large sample size of cases presenting with intra-amniotic infection will be necessary in order to be certain of the fetal origin of amniotic fluid neutrophils. We do not exclude the possibility, in some cases, that maternal leukocytes invade the amniotic cavity, given observations we have made, in which there is a severe maternal inflammatory response: a high amniotic fluid white blood cell count without evidence of a fetal inflammatory response (i.e. funisitis or chorionic vasculitis). The mechanisms responsible for neutrophil chemotaxis into the amniotic fluid probably rely on the production of neutrophil chemokines, such as IL-8 [59–62,138], GROα [66,67], and CXCL6 (granulocyte chemotactic protein-2) [63]. The fact that amniotic fluid neutrophils overexpressed TNF-α and MIP-1β suggests that their roles are different from those of other inflammatory cells. TNF-α has been implicated in the mechanisms of membrane rupture through the production of matrix metalloproteinases as well as in the induction of amniotic cell death [56,57,139–142]. MIP-1β is a monocyte chemokine and may, therefore, play a role in attracting neutrophils and monocytes in cases of intra-amniotic infection associated with bacteria [27]. IL-1α and IL-1β are also produced by neutrophils and have been implicated in the mechanisms of membrane rupture and parturition in general [38,39,143,144].

Amniotic fluid monocytes

Monocytes are derived from a common myeloid progenitor in the bone marrow and are then released into the peripheral blood, where they circulate for several days before infiltrating tissues and becoming tissue macrophages [145]. Monocytes have multiple functions due to their lack of defined specialization. Monocytes primarily serve to supplement tissue-resident macrophage populations, increasing response strength and sheer numbers during infection or injury [146]. This function is enhanced by the splenic monocyte reservoirs that release stored monocytes in an angiotensin-II-dependent manner [147]. Circulating monocytes are chemoattracted to both damaged and undamaged tissues where they undergo one of several fates [148,149]. First, monocytes can be activated to a pro-inflammatory state through signals such as lipopolysaccharide and IFN-γ, clearing pathogens and releasing factors similar to classical macrophages such as TNF-α, IL-1β, IL-12, ROS, and NO intermediates through inducible nitric oxide synthase (iNOS) [146]. Alternatively, at non-infectious sites

of injury, monocytes can acquire a repair-oriented phenotype through IL-4, IL-13, or IL-10 stimulation, increasing the release of homeostatic factors such as TGF-β [146]. Second, monocytes can replace decreased tissue-resident macrophage populations following injury-induced reduction, especially during infection [146,148]. Third, in addition to macrophage population renewal and function, monocytes can also differentiate into dendritic cells that migrate to the lymph nodes and other tissues [150]. Monocytes display morphological heterogeneity, including size variability, granularity, and nuclear morphology [146]. These innate cells were initially identified by their expression of large amounts of CD14 [148,151], a co-receptor for bacterial lipopolysaccharide [152]. Differential expression of CD14 and CD16 [153] allowed monocytes to be separated into two subsets: $CD14^{high}CD16^{-}$ and $CD14^{low}CD16^{+}$ cells [151]. $CD14^{high}CD16^{-}$ cells are known as classic monocytes [151], while $CD14^{low}CD16^{+}$ cells resemble tissue macrophages [154]. Therefore, the differential level expression of the CD14 molecule is a useful tool to distinguish monocytes from macrophages. Besides the differential expression of surface markers, monocytes are morphologically different from macrophages; therefore, histological examination is useful to confirm the state of differentiation of these innate immune cells [146, 155].

The study herein demonstrated that amniotic fluid monocytes are abundant in patients with clinical chorioamnionitis at term and with bacteria in the amniotic fluid. Our findings are consistent with a previous report, which detected monocytes in the amniotic fluid by using Wright staining [33]. We found that monocytes produce IL-1α, IL-1β, IL-8, MIP-1α, and MIP-1β. However, the predominant cytokines produced by monocytes, when compared to neutrophils, were IL-1α and IL-1β. These cytokines participate in the process of parturition [38,39,143,144] and in the host response to intra-amniotic infection [19, 38–42,50,156,157].

Strengths and limitations

This is the first study to characterize the phenotypic properties of leukocytes in intra-amniotic inflammation for patients diagnosed with clinical chorioamnionitis at term. These findings are novel and provide insight into the biology of the inflammatory response in the context of intra-amniotic infection. Future studies could examine other properties of neutrophils, such as the formation of NETs and the production of reactive oxygen radicals. The current studies are limited to patients at term, and future studies of patients who will undergo preterm labor can provide insight into the biology of intra-amniotic inflammation in preterm parturition.

Conclusions

Flow cytometry analysis of the amniotic fluid from patients diagnosed with intra-amniotic infection and clinical chorioamnionitis at term demonstrated that neutrophils and monocytes are the most common cells participating in the inflammatory process. We have characterized for the first time the differential cytokine expression by these cells in this important complication of pregnancy.

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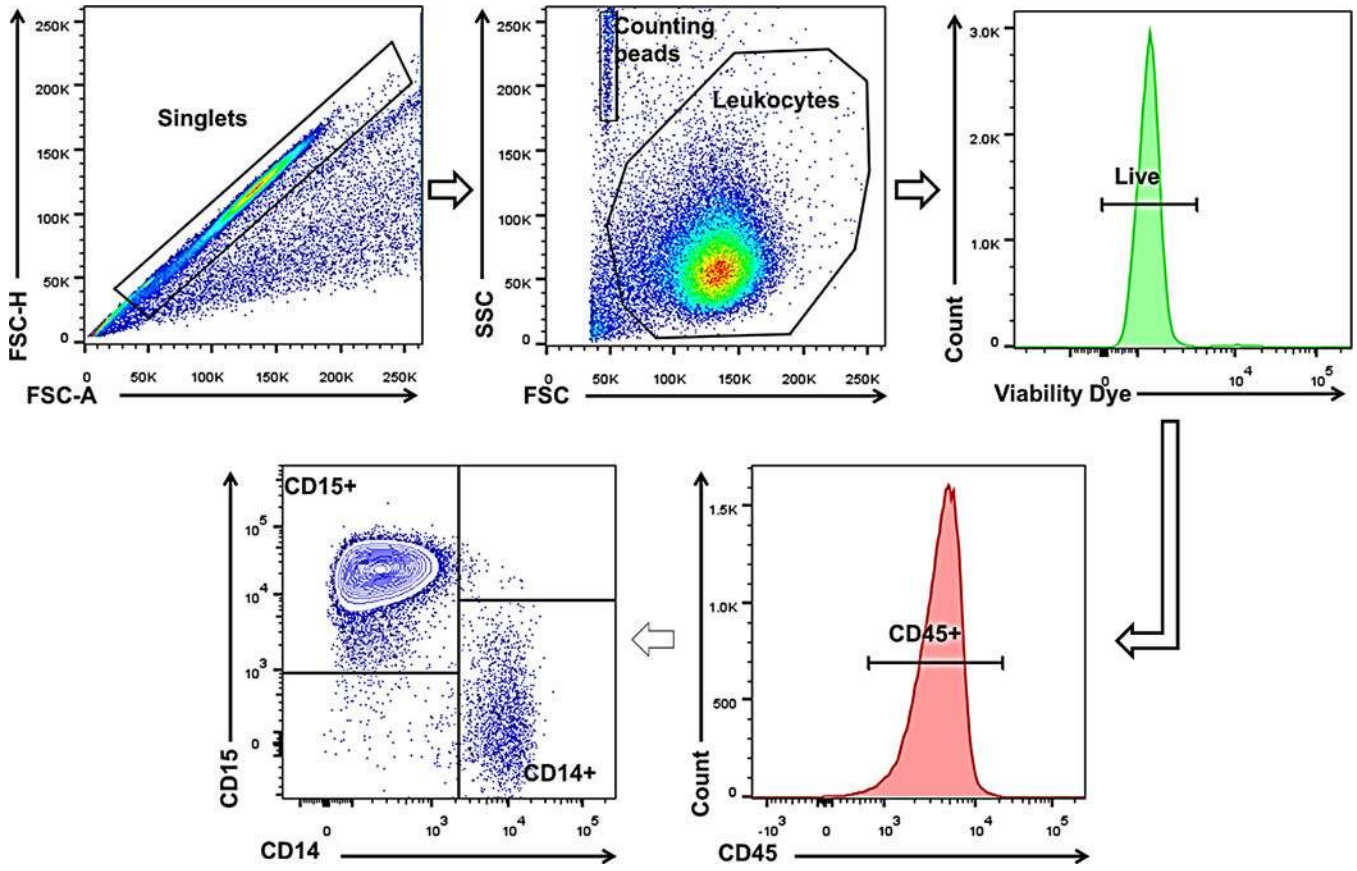

Figure 1.

Gating strategy used to identify amniotic fluid leukocytes in women with clinical chorioamnionitis at term. Total leukocytes (CD45+ cells) were gated using singlets, FSC vs. SSC and within the viability gate. Neutrophils (CD15+ cells) and monocytes (CD14+ cells) were gated within the CD45+ gate.

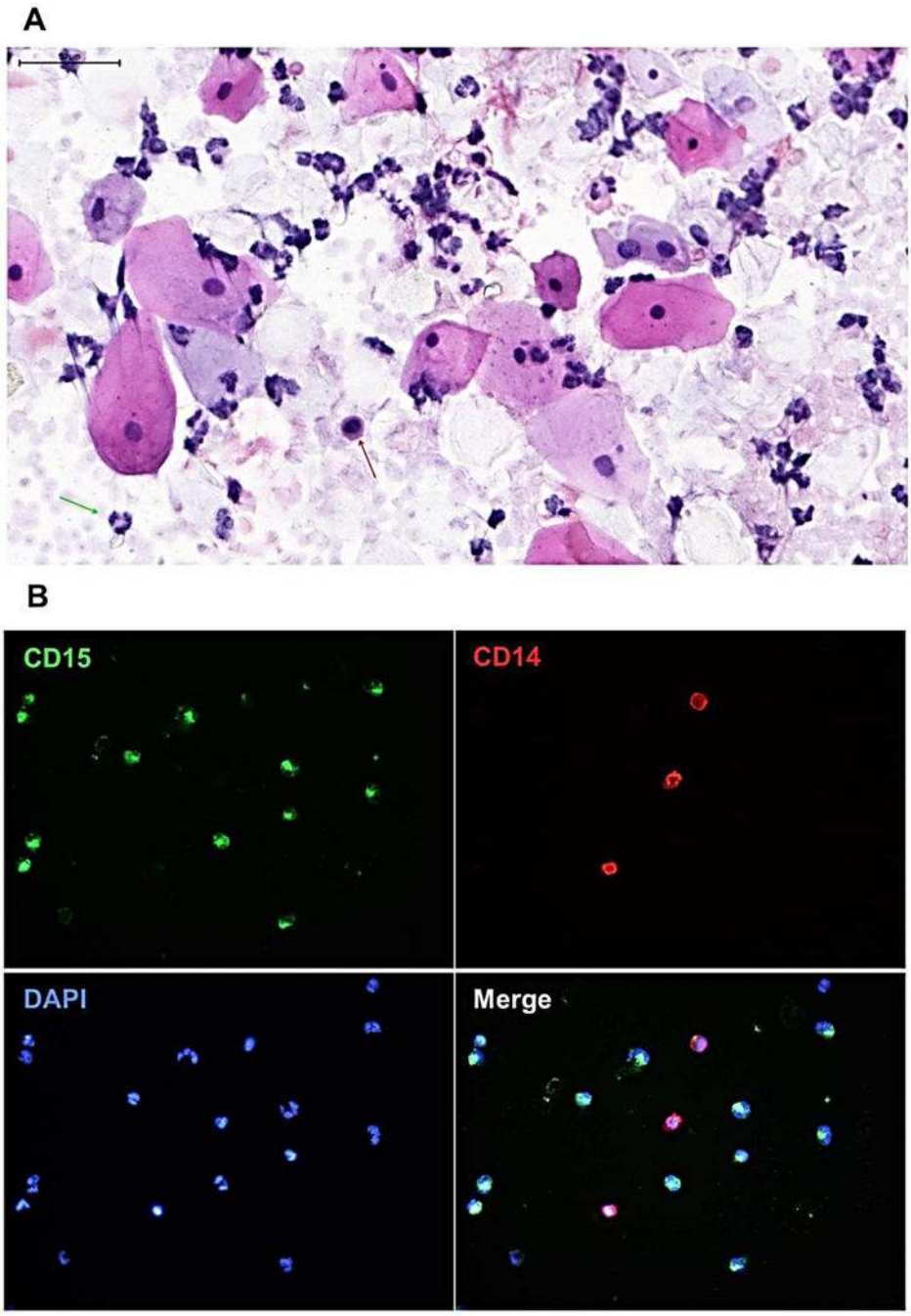

Figure 2.

Histological examination of amniotic fluid neutrophils and monocytes in women with clinical chorioamnionitis at term. A) Hematoxylin & Eosin staining shows the typical morphology of neutrophils (green arrow) and monocytes (red arrow) in the amniotic fluid of women with clinical chorioamnionitis at term. Magnification 400X. Scale bars: 50μm. B) Immunofluorescence of neutrophils and monocytes in the amniotic fluid of women with clinical chorioamnionitis at term. Neutrophils (CD15+ cells) are green, monocytes (CD14+ cells) are red, and nuclei are blue (DAPI). Magnification 400X.

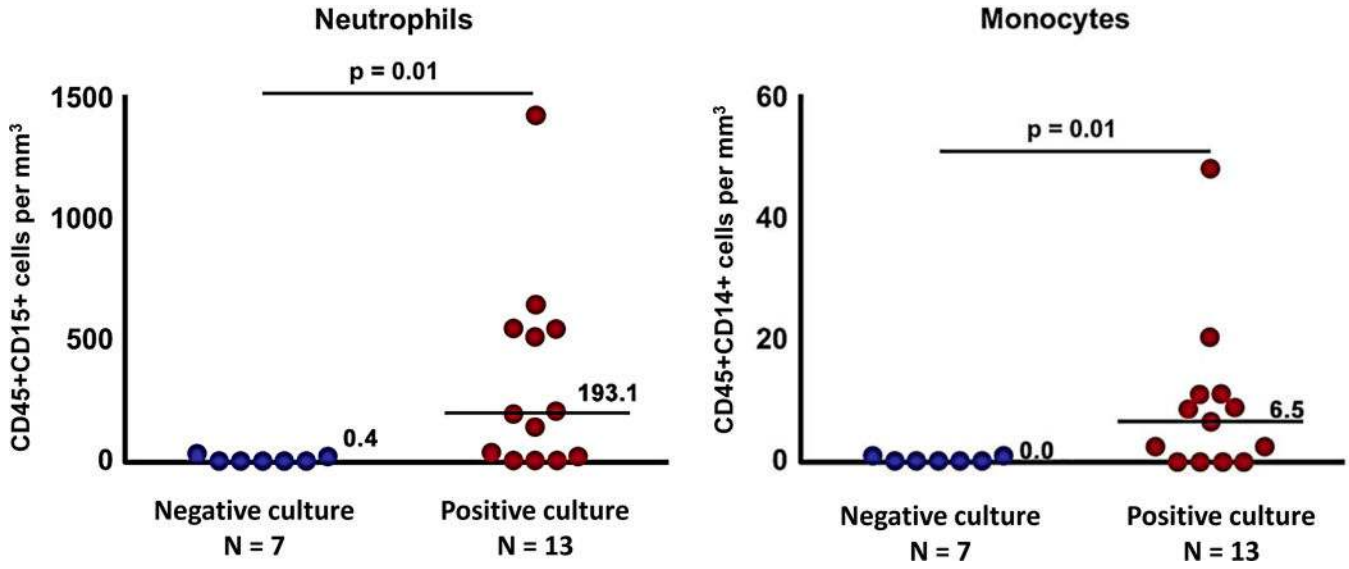

Figure 3.

Total number of amniotic fluid neutrophils and monocytes in women with clinical chorioamnionitis at term. Total number of neutrophils and monocytes in women with clinical chorioamnionitis at term with negative and positive cultures. Data are shown as scatter plots (median). Mann-Whitney U-tests.

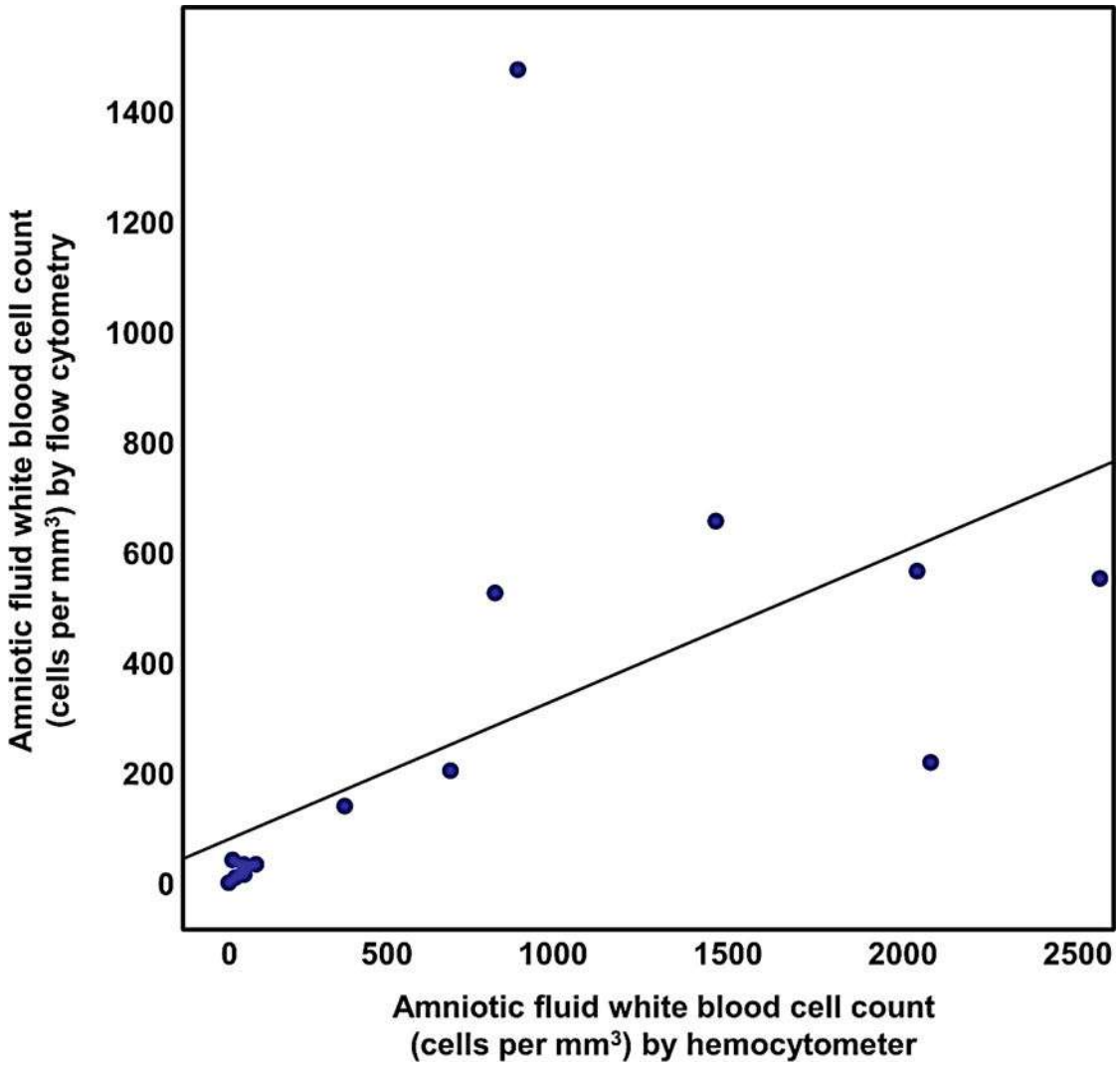

Figure 4.

Scatter plot of Spearman's correlation between amniotic fluid white blood cell counts determined by a hemocytometer and those determined by flow cytometry for patients with clinical chorioamnionitis at term. Black line indicates linear regression line.

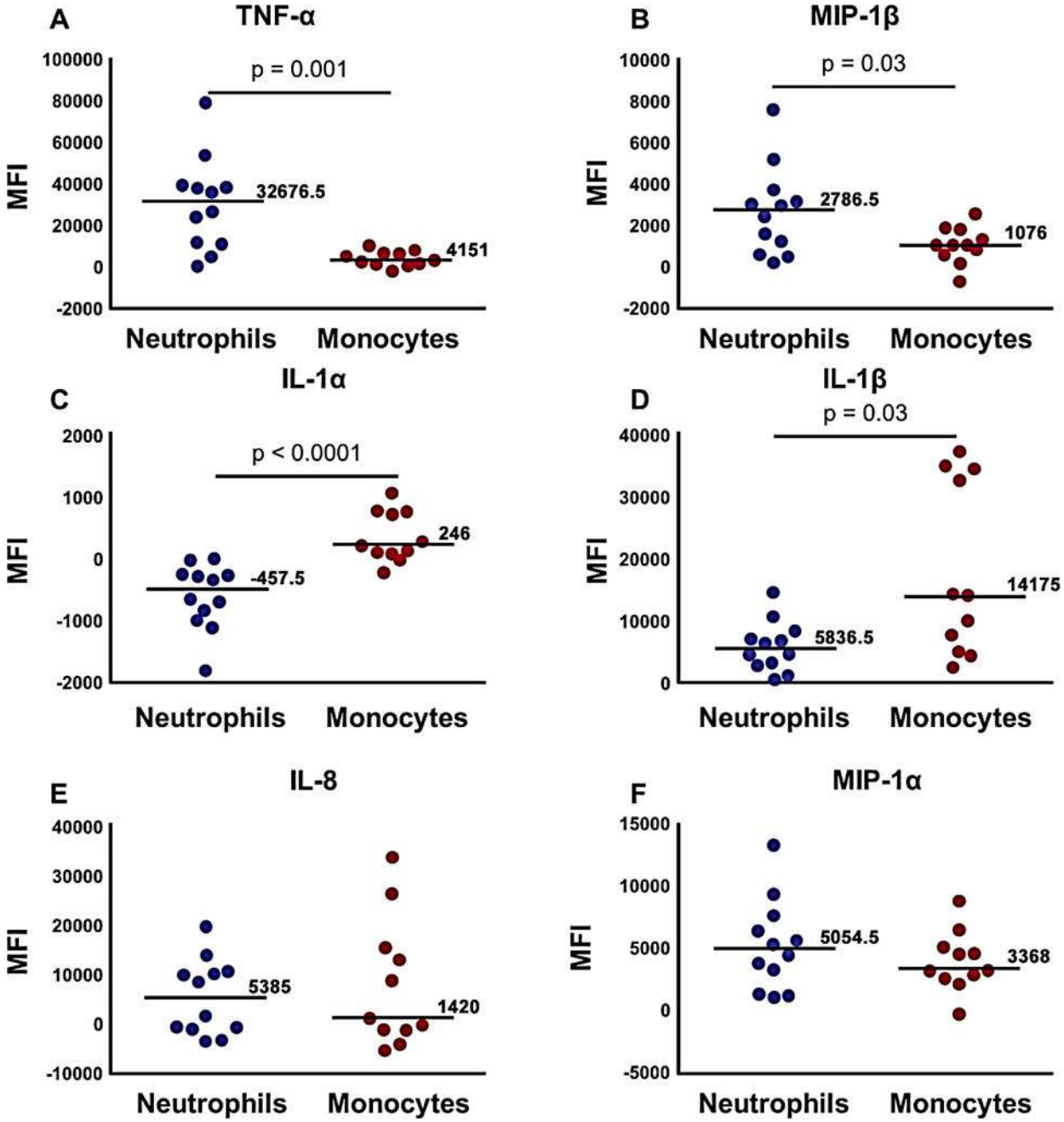

Figure 5.

Cytokine expression by amniotic fluid neutrophils and monocytes in women with clinical chorioamnionitis at term and positive cultures. The mean fluorescence intensity (MFI) of TNF-α (A), MIP-1β (B), IL-1α (C), IL-1β (D), IL-8 (E) and MIP-1α (F) was calculated as [MFI (anti-cytokine mAb) – MFI (isotype control mAb)]. Data are shown as scatter plots (median). Mann-Whitney U-tests.

Table 1

Clinical characteristics of the study population

	Negative amniotic fluid culture (n=7)	Positive amniotic fluid culture (n=13)	P-value
Maternal age (years)	25 (23–29)	21 (20–24)	0.08
Nulliparity	57.1% (4/7)	76.9% (10/13)	0.62
Body mass index (kg/m^2)	35.7 (32.1–36.5)	29.7 (25.225–30.7)	0.06
Gestational age at amniocentesis (weeks)	39.3 (38.9–39.8)	40.3 (38.1–40.9)	0.15
Rupture of membranes prior to amniocentesis	28.6% (2/7)	92.3% (12/13)	0.008
Epidural before amniocentesis	28.6% (2 /7)	92.3% (12 /13)	0.008
Conventional amniotic fluid analysis			
• Red blood cell count (cells/mm^3)	4 (4–131.5)	328 (128–2599)	0.02
• White blood cell count (cells/mm^3)	1 (0–8.5)	655 (19–1445)	0.01
• Glucose (mg/dL)	11 (4.5–17)	1 (1–1)	0.002
• Interleukin-6 (ng/mL)	1.1 (0.38–3.48)	13.2 (9–50.3)	0.02
Gestational age at delivery (weeks)	39.7 (39.3–40.1)	40.4 (38.1–40.9)	0.38
Vaginal delivery	71.4% (5/7)	61.5% (8/13)	1
Birthweight (grams)	3455 (3350–3687.5)	3445 (3000–3745)	0.70
Acute histologic chorioamnionitis	71.4% (5/7)	92.3% (12/13)	0.49
Acute funisitis	28.6% (2/7)	92.3% (12/13)	0.008

Data presented as median (interquartile range) or % (n).

Table 2

Amniotic fluid and placental characteristics of patients with clinical chorioamnionitis at term who had positive amniotic fluid cultures

Case Number	Gestational Age at Amniocentesis (weeks)	Clinical Laboratory		Flow Cytometry		Microorganisms in Amniotic Fluid	Acute Histologic Chorioamnionitis[a]	Acute Funisitis
		Total amniotic fluid white blood cell count (cells/mm^3)	Amniotic fluid IL-6 (ng/mL)	Neutrophils (CD15 cells/mm^3)	Monocytes (CD14 cells/mm^3)			
1	37.9	864	1.91	1427.2	48.1	*Gardnerella vaginalis, Mycoplasma hominis, Peptostreptococcus, Streptococcus viridans, Ureaplasma urealyticum, Porphyromonas*	Stage 2	Stage 1
2	41.3	19	41.6	18.4	0.3	*Ureaplasma urealyticum, Streptococcus anginosus*, Beta hemolytic *streptococcus*	Stage 2	Stage 1
3	37.1	1445	131.8	647.2	10.9	*Mycoplasma hominis, Streptococcus* group B, *Ureaplasma urealyticum*	Stage 2	Stage 1
4	41.6	655	50.3	193.1	6.5	*Mycoplasma hominis, Ureaplasma urealyticum*	Stage 2	Stage 1
5	40.9	2053	13.2	547.6	20.4	*Lactobacillus Jensenii*	Stage 2	Stage 1
6	40.6	0	1.39	0.4	0.0	*Mycoplasma hominis, Ureaplasma urealyticum*	Stage 1	Stage 1
7	40.3	790	18.4	513.3	10.9	*Gardnerella vaginalis, Actinomyces israelii, Enterococcus faecalis*	Stage 2	Stage 2
8	39.9	0	1.49	0.0	0.0	*Streptococcus group B*	Stage 2	Stage 2
9	38.1	70	11.48	28.1	2.4	*Ureaplasma urealyticum*	None	None
10	38	2090	9	210.7	8.4	*Ureaplasma urealyticum*	Stage 2	Stage 1
11	40.9	2600	51.1	543.8	9.3	*Ureaplasma urealyticum, Candida albicans, Candida Glabrata, Candida tropicalis, Klebsiella*	Stage 1	Stage 1
12	40.1	9	10.5	4.5	0.1	*Streptococcus* group B	Stage 2	Stage 1
13	41.1	340	51.3	141.4	2.2	*Ureaplasma urealyticum*	Stage 2	Stage 1

[a]Acute histologic chorioamnionitis: Stage 1, early, acute subchorionitis/chorionitis; Stage 2, intermediate, acute chorioamnionitis

[b]Funisitis: Stage 1, early umbilical phlebitis/chorionic vasculitis; Stage 2, intermediate, umbilical arteritis

Table 3

The relationship of the white blood cell counts between flow cytometry and the standard techniques, as well as the amniotic fluid IL-6 concentrations, in patients with clinical chorioamnionitis at term.

Correlation between	Correlation Coefficient (Spearman's correlation)	P-value
Total white blood cell count (cells/mm^3) by hemocytometer and total white blood cell count by flow cytometry	0.88	<0.001
Neutrophils (CD15 cells/mm^3) and monocytes (CD14 cells/mm^3) by flow cytometry	0.97	<0.001
Amniotic fluid interleukin-6 concentration (ng/mL) and		
• Neutrophils (CD15 cells/mm^3) by flow cytometry	0.59	0.007
• Monocytes (CD14 cells/mm^3) by flow cytometry	0.61	0.004
• Total white blood cell count (cells/mm^3) by flow cytometry	0.59	0.007