

DR NARDHY GOMEZ-LOPEZ (Orcid ID : 0000-0002-3406-5262)

DR ROBERTO ROMERO (Orcid ID : 0000-0002-4448-5121)

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## **Gasdermin D: Evidence of Pyroptosis in Spontaneous Preterm Labor with Sterile Intra-amniotic Inflammation or Intra-amniotic Infection**

Nardhy Gomez-Lopez<sup>1-3</sup>, Roberto Romero<sup>1,4-6</sup>, Adi L. Tarca<sup>1,2</sup>, Derek Miller<sup>1,2</sup>, Bogdan Panaitescu<sup>1,2</sup>, George Schwenkel<sup>1,2</sup>, Dereje W. Gudicha<sup>1,2</sup>, Sonia S. Hassan<sup>1,2,7</sup>, Percy Pacora<sup>1,2</sup>, Eunjung Jung<sup>1,2</sup>, Chaur-Dong Hsu<sup>1,2,7</sup>

<sup>1</sup>Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services, Bethesda, Maryland, and Detroit, Michigan, USA

<sup>2</sup>Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan, USA

<sup>3</sup>Department of Immunology, Microbiology and Biochemistry, Wayne State University School of Medicine, Detroit, Michigan, USA

<sup>4</sup>Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan, USA

<sup>5</sup>Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, Michigan, USA

<sup>6</sup>Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA

<sup>7</sup>Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan, USA

**Short title:** Pyroptosis in spontaneous preterm labor with intra-amniotic inflammation

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**Address correspondence to:**

Nardhy Gomez-Lopez, MSc, PhD, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Perinatology Research Branch, NICHD/NIH/DHHS, 275 E. Hancock, Detroit, Michigan 48201, USA, Tel (313) 577-8904, Email: nardhy.gomez-lopez@wayne.edu; ngomezlo@med.wayne.edu.

Roberto Romero, MD, D. Med. Sci. Perinatology Research Branch, NICHD/NIH/DHHS, Hutzel Women's Hospital, 3990 John R, Box 4, Detroit, Michigan, 48201, USA, Telephone: (313) 993-2700, Fax: (313) 993-2694, Email: prbchiefstaff@med.wayne.edu

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**ABSTRACT**

**Problem:** Pyroptosis, inflammatory programmed cell death, is initiated through the inflammasome and relies on the pore-forming actions of the effector molecule gasdermin D. Herein, we investigated whether gasdermin D is detected in women with spontaneous preterm labor and sterile intra-amniotic inflammation or intra-amniotic infection.

**Method of study:** Amniotic fluid samples (n = 124) from women with spontaneous preterm labor were subdivided into the following groups: 1) those who delivered at term (n = 32); and those who delivered preterm 2) without intra-amniotic inflammation (n = 41), 3) with sterile intra-amniotic inflammation (n = 32), or 4) with intra-amniotic infection (n = 19), based on amniotic fluid IL-6 concentrations and microbiological status of amniotic fluid (culture and PCR/ESI-MS). Gasdermin D concentrations were measured using an ELISA kit. Multiplex immunofluorescence staining was also performed to determine the expression of gasdermin D, caspase-1, and interleukin-1 $\beta$  in the chorioamniotic membranes. Detection of pyroptosis (active caspase-1) in decidual cells from women with preterm labor and birth was performed using flow cytometry.

**Results:** 1) Gasdermin D was detected in the amniotic fluid and chorioamniotic membranes from women who underwent spontaneous preterm labor/birth with either sterile intra-amniotic inflammation or intra-amniotic infection, but was rarely detected in those without intra-amniotic inflammation. 2) Amniotic fluid concentrations of gasdermin D were higher in women with intra-amniotic infection than in those with sterile intra-amniotic inflammation, and its expression in the chorioamniotic membranes was associated with caspase-1 and IL-1 $\beta$  (inflammasome mediators). 3) Decidual stromal cells and leukocytes isolated from women with preterm labor and birth are capable of undergoing pyroptosis since they expressed active caspase-1.

**Conclusions:** Pyroptosis can occur in the context of sterile intra-amniotic inflammation and intra-amniotic infection in patients with spontaneous preterm labor and birth.

**Keywords:** amniotic fluid, chorioamniotic membranes, caspase-1, interleukin-1beta, inflammasome, parturition, pregnancy, prematurity, fetal inflammatory response, funisitis, chorioamnionitis, microbial invasion of the amniotic cavity.

## INTRODUCTION

Preterm birth, the leading cause of perinatal morbidity and mortality worldwide<sup>1-3</sup>, is often preceded by spontaneous preterm labor<sup>4-8</sup>, a syndrome of multiple etiologies<sup>9</sup>. Among the proposed causes of preterm labor, intra-amniotic inflammation represents the only causal link to preterm birth<sup>10-22</sup>. Intra-amniotic inflammation can result from microbial invasion of the amniotic cavity, referred to as intra-amniotic infection (IAI), or

can occur in the absence of detectable microorganisms using both culture and molecular microbiological techniques, known as sterile intra-amniotic inflammation (SIAI) <sup>23-27</sup>. Such an inflammatory process is commonly observed in women with preterm labor and intact membranes <sup>24</sup>, those with an asymptomatic short cervix <sup>25</sup>, and those with preterm prelabor rupture of membranes <sup>27</sup>. Indeed, we have provided solid evidence indicating that alarmins (i.e. molecules that trigger sterile inflammation <sup>28-30</sup>) are associated with intra-amniotic inflammation and preterm labor and birth <sup>31-36</sup>. In addition, systemic <sup>37,38</sup> or intra-amniotic <sup>39,40</sup> administration of alarmins induces preterm labor and birth in mice. The mechanisms whereby alarmins and microbes induce such inflammation involve the inflammasome <sup>40-50</sup>.

The inflammasome is a cytoplasmic multi-subunit protein complex that, once assembled, catalyzes the activation of caspase-1 <sup>51-69</sup> and the consequent release of the mature forms of the inflammatory cytokines IL-1 $\beta$  and/or IL-18 <sup>70-78</sup>. At the cellular level, the end result of inflammasome activation is pyroptosis, an inflammatory type of programmed cell death that is characterized by the uncontrolled release of cytosolic contents <sup>79-82</sup>. A primary effector molecule of pyroptosis is gasdermin D, a protein that is cleaved by the active forms of caspase-1 and caspase-11 <sup>83,84</sup>. Once cleaved into its active fragments, gasdermin D forms pores in the cell membrane <sup>85-88</sup>, inducing pyroptotic cell death and allowing the release of cytosolic proteins, including inflammatory mediators such as IL-1 $\beta$  <sup>80,85</sup>. Importantly, cleaved gasdermin D is also released into the extracellular space <sup>89</sup>. Thus, gasdermin D is an important component of inflammasome activation-induced pathological inflammation and its detection in biological fluids can serve as an *in vivo* readout of pyroptosis. Although we have shown that inflammasome activation occurs during the pathological inflammatory process of preterm labor <sup>40,44,46,47</sup>, no evidence implicating pyroptosis has been generated. Herein, we investigated whether spontaneous preterm labor in the setting of sterile intra-amniotic inflammation or intra-amniotic infection is accompanied by an increase in gasdermin D concentrations as an *in vivo* indicator of pyroptosis. **MATERIALS AND**

## **METHODS**

### **Study population**

This is a cross-sectional study that included the following patients: (1) women with spontaneous preterm labor that delivered at term with a negative amniotic fluid culture and an IL-6 concentration  $<2.6$  ng/mL ( $n = 32$ ); (2) women with spontaneous preterm labor who delivered preterm without IAI or SIAI ( $n = 41$ ); (3) women with spontaneous preterm labor who delivered preterm with SIAI ( $n = 32$ ); and (4) women with spontaneous preterm labor who delivered preterm with IAI ( $n = 19$ ) (see diagnostic criteria below). Chorioamniotic membranes samples from women in each of the study groups were also studied using multiplex immunofluorescence and phenoptics. Decidua tissues (decidua parietalis attached to the chorioamniotic membranes) from women with preterm labor were used for the detection of active caspase-1 by flow cytometry ( $n = 5$ ). Women with multiple gestations or those who had fetuses affected with chromosomal and/or sonographic abnormalities were excluded. Maternal and neonatal data were obtained by retrospective clinical chart review. Amniotic fluid samples were retrieved from the bank of biological specimens at the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), U.S. Department of Health and Human Services (DHHS), Detroit, Michigan. All women provided written informed consent prior to the collection of samples, and the Institutional Review Boards of Wayne State University and the NICHD approved the collection and use of materials and clinical data for research purposes.

### **Clinical definitions**

Gestational age was determined by the last menstrual period and confirmed by ultrasound examination or by ultrasound examination alone if the sonographic determination of gestational age was inconsistent with menstrual dating by more than 1 week. Spontaneous preterm labor was defined as the presence of regular uterine contractions with a frequency of at least 2 every 10 min and cervical changes between 20 and 36 (6/7) weeks of gestation. Microbial invasion of the amniotic cavity (MIAC) was defined as a positive amniotic fluid culture and/or a polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology—Athogen, Carlsbad, CA, USA) test result <sup>23,90-93</sup>. Intra-amniotic inflammation was defined as an

amniotic fluid IL-6 concentration  $\geq 2.6$  ng/mL<sup>94-98</sup>. SIAI was defined as an amniotic fluid IL-6 concentration  $\geq 2.6$  ng/mL<sup>94</sup> without microorganisms detected by culture or PCR/ESI-MS<sup>23-27</sup>. IAI (or microbial-associated intra-amniotic inflammation) was defined as the presence of MIAC with intra-amniotic inflammation<sup>23-27,99-101</sup>. Neonatal Apgar scores at 1 and 5 minutes were determined using previously established criteria<sup>102-104</sup>. Acute histologic chorioamnionitis and acute funisitis were diagnosed according to established criteria<sup>105-107</sup>.

### **Amniotic fluid sample collection**

Amniotic fluid samples were obtained by transabdominal amniocentesis under antiseptic conditions and monitored by ultrasound. Transabdominal amniocentesis was performed for the detection of intra-amniotic inflammation and/or infection. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe and centrifuged at 1,300 X g for 10 min at 4°C, and the supernatant was stored at -80°C until use. A portion of this amniotic fluid was also transported to the clinical laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical tests also included the determination of amniotic fluid white blood cell count<sup>108</sup>, glucose concentration<sup>109</sup>, Gram stain<sup>110</sup>, and IL-6 concentration<sup>94,111</sup>.

### **Placental histopathological evaluation**

Placentas were examined histologically by perinatal pathologists blinded to clinical diagnoses and obstetrical outcomes. Briefly, three to nine sections of the placenta were examined, and at least one full-thickness section was taken from the center of the placenta; other sections were taken randomly from the placental disc. Acute and chronic inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading<sup>105-107,112,113</sup>.

### **Determination of gasdermin D concentrations in the amniotic fluid**

Concentrations of gasdermin D in amniotic fluid samples were determined by using a sensitive and specific enzyme-linked immunosorbent assay (ELISA) kit obtained

from MyBioSource (Cat#MBS9338251; San Diego, CA, USA). This ELISA kit was initially validated in our laboratory prior to the execution of this study. Amniotic fluid concentrations of gasdermin D were obtained by interpolation from the standard curve. The inter- and intra-assay coefficients of variation were 12.757% and 11.249%, respectively. The sensitivity of the assay was 0.249 ng/mL.

### **Multiplex immunofluorescence and phenoptics**

Five- $\mu$ m-thick sections of formalin-fixed, paraffin-embedded chorioamniotic membranes (amnion and choriondecidua) of patients from our study groups were cut and mounted on SuperFrost Plus microscope slides. Multiplex immunofluorescence staining was performed using the Opal 7 kit (Cat#NEL811001KT; PerkinElmer, Waltham, MA), according to the manufacturer's instructions. Prior to multiplex immunofluorescence staining, each analyte was individually optimized with single antibody staining combined with different fluorescent TSA® reagents (PerkinElmer). After deparaffinization, slides were placed in antigen retrieval (AR) buffer and boiled using a microwave oven. Following blocking to eliminate nonspecific binding, slides were incubated with antibodies against gasdermin D (Cat#20770-1-AP; Proteintech, Rosemont, IL, USA), caspase-1 (Cat#MA5-16215; Invitrogen, Rockford, IL), or IL-1 $\beta$  (Cat#NBP1-19775; Novus Biologicals, Littleton, CO) at room temperature. The slides were then washed and incubated with Opal Polymer HRP Ms+Rb (Cat#ARH1001EA; PerkinElmer). Next, the slides were incubated with one of the following fluorescent TSA® reagents included in the Opal 7 kit to detect each antibody staining: Opal 520, Opal 570, or Opal 690 (dilution 1:100). After washing, the slides were counterstained with Spectral DAPI (Cat#FP1490; PerkinElmer) and mounted using ProLong Diamond Antifade Mountant (Life Technologies, Eugene, OR). Autofluorescence slides as well as slides stained with isotype (negative controls) were included. Multiplex staining was performed by consecutively staining slide-mounted tissues using the same antibody concentrations and conditions validated through singleplex staining. Each previous primary and secondary antibody was removed by boiling in AR buffer before the application of the next primary antibody. After multiplex staining, the slides were imaged using the Vectra Polaris Multispectral Imaging System (PerkinElmer) and images were analyzed and

converted to immunohistochemistry view using the InForm 2.4.1 image analysis software (PerkinElmer).

### **Determination of active caspase-1 in decidual cells using flow cytometry**

Decidual cells were isolated from the decidua parietalis as previously described<sup>114</sup>, with minor modifications. Briefly, the decidua parietalis was separated from the chorioamniotic membranes, and the decidual tissues were homogenized using a gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA) in StemPro Accutase Cell Dissociation Reagent (Life Technologies, Grand Island, NY, USA). Homogenized tissues were incubated for 45 min at 37°C with gentle agitation. After incubation, tissues were washed in sterile 1X phosphate-buffered saline (PBS) (Life Technologies) and filtered through a 100 µm cell strainer (Falcon, Corning Life Sciences Inc., Durham, NC, USA). The resulting cell suspension was centrifuged at 300 x g for 10 min at 4°C, resuspended in 5 mL of 1X PBS, and gently layered over 5 mL of Polymorphprep (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). The cell suspension/Polymorphprep gradient was then centrifuged at 500 x g and 4°C for 30 min, after which the mononuclear cell layer was carefully pipetted into a clean tube and washed in 1X PBS at 300 x g for 10 min at 4°C. The cells were then resuspended in 100 µL of stain buffer (BD Biosciences, San Jose, CA, USA) and the following extracellular fluorochrome-conjugated monoclonal anti-human antibodies were added to the cell suspension: CD45-AlexaFluor700 (clone H130, cat# 560566, BD Biosciences), CD15-BV650 (clone H198, cat# 564232, BD Biosciences), CD14-BV605 (clone M5E2, cat# 301833, BioLegend), CD3-APC-Cy7 (clone SK7, cat#557832, BD Biosciences), and CD19-PE-Cy5 (clone HIB19, cat# 555414, BD Biosciences). The cells were then incubated at 4°C for 30 min in the dark and washed in stain buffer at 300 x g for 10 min to remove excess antibody, followed by a second wash in 1X PBS. The cells were resuspended in 290 µL of 1X PBS and immediately used for intracellular active caspase-1 staining.

Detection of active caspase-1 to determine pyroptosis was performed using the FAM-FLICA Caspase-1 Assay Kit (cat# 97, ImmunoChemistry Technologies, Bloomington, MN, USA), following the manufacturers' instructions. Briefly, a 30X FAM-

FLICA solution was prepared in 1X PBS and 10  $\mu$ L of the prepared solution were added to the cell suspension. The cells were incubated at 37°C for 30 min followed by two washes in 1X apoptosis wash buffer (ImmunoChemistry Technologies) at 300 x g for 10 min. After the second wash, red blood cell lysis was performed by resuspending the cells in 1 mL of 0.8% ammonium chloride solution (STEMCELL Technologies, Cambridge, MA, USA) and incubating the cells for 5 min at 37°C. The cell suspension was then washed in stain buffer at 300 x g for 10 min and resuspended in 0.5 mL of stain buffer. Finally, immediately prior to flow cytometer acquisition, 1  $\mu$ L of 4',6-diamidino-2-phenylindole (DAPI) solution (1  $\mu$ g/mL, cat# D9542, Sigma-Aldrich, St Louis, MO, USA) was added to the cell suspension. The cells were acquired using the BD LSRFortessa Flow Cytometer (BD Biosciences) and BD FACSDiva 6.0 software (BD Biosciences). The analysis and figures were performed using FlowJo software version 10 (FlowJo, LLC, Ashland, OR, USA). Active caspase-1 and DAPI-positive cells were considered to be pyroptotic.

### **Statistical analysis**

Data were analyzed using IBM SPSS version 19.0 software (IBM Corporation; Armonk, NY, USA) and R statistical language and environment ([www.r-project.org](http://www.r-project.org)). For patient demographics, the Fisher's exact test was used to compare proportions between groups and the Mann-Whitney U-test was used for comparing continuous variables between groups. To determine the association between amniotic fluid gasdermin D concentrations and the presence of SIAI or IAI, the number of women with an amniotic fluid gasdermin D concentration above the limit of detection (LOD, 0.249 ng/mL) was determined for each study group. The associations between amniotic fluid gasdermin D concentrations > 0.249 ng/mL and the presence of SIAI or IAI were determined using a Chi-square test for proportions, with relative risk estimates and p-values obtained using the *epiR* package in R. The Wilcoxon signed rank test was also used to compare gasdermin D concentrations between the two groups in which concentrations were above the detection limit for a majority of patients. Statistical analyses for gasdermin D included adjustment for gestational age at sampling. A p-value <0.05 was considered significant.

## RESULTS

### Characteristics of the study population

Amniotic fluid samples from 124 patients with spontaneous preterm labor were included in this study. The demographic and clinical characteristics of the study population are displayed in Table 1. Neonates whose mother had spontaneous preterm labor and delivered preterm with either SIAI or IAI had lower median birthweights and Apgar scores at 1 and 5 minutes compared to the other study groups (Table 1). The prevalence of acute histological chorioamnionitis and acute funisitis was both significantly different among the study groups, with the highest frequency occurring in patients with spontaneous preterm labor that delivered preterm with SIAI or IAI (Table 1).

### Amniotic fluid gasdermin D concentrations in women with spontaneous preterm labor

Amniotic fluid gasdermin D concentrations above the limit of detection (LOD, 0.249 ng/mL) were determined in each study group as shown in Table 2. Few patients with spontaneous preterm labor that delivered either at term or preterm without SIAI or IAI had amniotic fluid gasdermin D concentrations above the LOD [preterm labor who delivered at term: 3.1% (1/32); preterm labor who delivered preterm without SIAI or IAI: 4.9% (2/41)] (Table 2). Fifty percent (16/32) of patients with spontaneous preterm labor that delivered preterm with SIAI had amniotic fluid gasdermin D concentrations above the LOD, whereas 89.5% (17/19) of patients who underwent spontaneous preterm labor and delivered preterm with IAI had an elevated gasdermin D concentration (Table 2).

No differences were observed in the frequency of gasdermin D detection between patients with spontaneous preterm labor and birth without intra-amniotic inflammation and those with spontaneous preterm labor who delivered at term without intra-amniotic inflammation [relative risk (RR) 1.2,  $p = 0.7$ ] (Table 3). We then examined the association between gasdermin D concentrations and the presence of SIAI or IAI in patients with spontaneous preterm labor and birth. The frequency of detected amniotic fluid gasdermin D was higher in patients with spontaneous preterm labor and birth with either SIAI (RR 2.76,  $p < 0.001$ ) or IAI (RR 15.58,  $p < 0.001$ ) compared to those with

preterm labor who delivered at term (Table 3). Moreover, the frequency of detected amniotic fluid gasdermin D was higher in patients with spontaneous labor and birth with either SIAI (RR 3.06,  $p < 0.001$ ) or IAI (RR 18.34,  $p < 0.001$ ) compared to those with preterm labor and birth without intra-amniotic inflammation (Table 3). Importantly, the frequency of detected amniotic fluid gasdermin D (RR 4.64,  $p = 0.004$ , Table 3), as well as its concentration ( $p < 0.001$ , Figure 1), was higher in patients with spontaneous preterm labor and birth with IAI compared to those with SIAI. These results indicate that intra-amniotic inflammation induced either by microbes (IAI) or alarmins (SIAI) is significantly associated with the presence of gasdermin D, the effector molecule of pyroptosis, in amniotic fluid of patients with spontaneous preterm labor and birth.

### **Gasdermin D in the chorioamniotic membranes of women with spontaneous preterm labor**

The co-expression of gasdermin D, caspase-1, and IL-1 $\beta$  was low in the chorioamniotic membranes of women who underwent spontaneous preterm labor and delivered at term or preterm without intra-amniotic inflammation, and there were no evident differences in expression of these molecules between these two study groups (Figure 2). Yet, gasdermin D was highly expressed in the chorioamniotic membranes of patients with spontaneous preterm labor who delivered preterm either with SIAI or IAI (Figure 3). Moreover, gasdermin D expression in these study groups was detected along with caspase-1 and IL-1 $\beta$  in the chorioamniotic membranes, which is likely indicative of inflammasome-mediated pyroptosis (Figure 3). Additional experiments exploring the expression of active caspase-1 by non-leukocytes and leukocytes isolated from the decidual tissues of women with spontaneous preterm labor were also performed. We report that decidual cells from women with preterm labor and birth can undergo pyroptosis since such cells expressed active caspase-1 and had a permeable cell membrane (DAPI+ cells) (Figure 4A). The main leukocyte populations expressing active caspase-1 were macrophages and neutrophils (Figure 4B). Decidual T cells and B cells expressed minimal or no detectable active caspase-1, respectively (data not shown). These findings suggest that pyroptosis can occur in the chorioamniotic membranes and decidual tissues of women with spontaneous preterm labor and birth.

## DISCUSSION

### Principal findings of the study

Herein, we report that: 1) extracellular gasdermin D is commonly detected in the amniotic fluid of women who underwent spontaneous preterm labor/birth with either sterile intra-amniotic inflammation or intra-amniotic infection, and was rarely detected in those without intra-amniotic inflammation; 2) both the frequency of detected amniotic fluid gasdermin D and its concentration were higher in women with intra-amniotic infection than in those with sterile intra-amniotic inflammation; 3) gasdermin D was highly expressed in the chorioamniotic membranes of patients with either sterile intra-amniotic inflammation or intra-amniotic infection, and was associated with the inflammasome mediators caspase-1 and IL-1 $\beta$ ; and 4) non-leukocytes and leukocytes (e.g. macrophages and neutrophils) expressed active caspase-1 in the decidua of women with preterm labor and birth. Collectively, these findings show that pyroptosis can occur in the amniotic cavity, chorioamniotic membranes, and decidua of women with spontaneous preterm labor and birth.

### Pyroptosis in spontaneous preterm labor with intra-amniotic inflammation induced by microbes

Herein, we found that an increased frequency of detected extracellular gasdermin D in amniotic fluid is significantly associated with the presence of intra-amniotic infection in patients with spontaneous preterm labor and birth. Previous studies have demonstrated a clinical role for gasdermin D as a marker for pyroptosis both in affected tissues, as shown in the livers of patients with alcoholic hepatitis<sup>115</sup> as well as in biological fluids such as plasma in patients with acute respiratory distress syndrome and sepsis<sup>116</sup>. In the intra-amniotic cavity, the cells present in this compartment<sup>117,118</sup> are a possible source of gasdermin D, particularly the innate immune cells (i.e. neutrophils and monocytes/macrophages) that are increased in women with intra-amniotic infection<sup>108,117-122</sup>. Gasdermin D has a dual purpose in neutrophils, forming pores which allow for the release of IL-1 $\beta$  during pyroptosis<sup>89,123-125</sup> but also partially mediating the release of neutrophil extracellular traps (NETs)<sup>126-128</sup>, which can be found in the amniotic cavity and chorioamniotic membranes of women with intra-amniotic

infection<sup>122,129</sup>. Macrophages, in which pyroptosis was first described and characterized<sup>79,80,130</sup>, have also been shown to display gasdermin D-mediated secretion of IL-1 $\beta$  in response to microbes or their products<sup>83,84,125,131,132</sup>. Moreover, keratinocytes, a major cellular component of amniotic fluid<sup>133-137</sup>, have been shown to express inflammasome components and mediators such as IL-1 $\beta$ <sup>138,139</sup>. Thus, both innate immune cells and epithelial cells may contribute to amniotic fluid concentrations of gasdermin D in women who underwent spontaneous preterm labor with intra-amniotic infection. Yet, additional experimentation is required to investigate the origin of gasdermin D in amniotic fluid.

A possibility is that the adaptive immune cells (T cells) in amniotic fluid may also release gasdermin D. This is supported by the fact that T cells undergo caspase-1-mediated pyroptosis in response to viral infections such as HIV<sup>140-143</sup>, although gasdermin D expression was not demonstrated in these studies. Further research is needed to investigate whether T cells release gasdermin D as a mechanism of pyroptosis in the amniotic cavity of women with spontaneous preterm labor and intra-amniotic infection.

Amniotic fluid immune cells can release gasdermin D; yet, the chorioamniotic membranes and placenta may be the primary sources of this pyroptosis effector molecule in the amniotic cavity. Herein, we show that the expression of gasdermin D is highest in the chorioamniotic membranes of patients with preterm labor and intra-amniotic infection and that decidual cells including leukocytes (macrophages and neutrophils) are capable of expressing active caspase-1. This is in line with previous work demonstrating that patients with spontaneous preterm labor with acute histologic chorioamnionitis, a placental lesion associated with intra-amniotic infection<sup>105,106,144-151</sup>, have elevated expression of inflammasome components and mediators in the chorioamniotic membranes<sup>44</sup>. Furthermore, the ultrasound-guided intra-amniotic administration of a microbial product, lipopolysaccharide, induces inflammasome activation in the murine fetal membranes, indicating that pyroptosis occurs in the amniotic cavity prior to preterm birth<sup>47</sup>. In the clinical setting, inflammasome activation and pyroptosis could also be triggered by genital mycoplasmas<sup>152-155</sup>, the most commonly found bacteria in women with preterm labor and intra-amniotic infection<sup>23,156-162</sup>. Together, these findings implicate inflammasome-mediated pyroptosis in the

mechanisms that lead to an intra-amniotic inflammatory response in patients with spontaneous preterm labor with microbial invasion of the amniotic cavity.

### **Pyroptosis in spontaneous preterm labor with sterile intra-amniotic inflammation**

In the current study, we report that women with spontaneous preterm labor and sterile intra-amniotic inflammation had a greater frequency of detected amniotic fluid gasdermin D; yet, this was lower than in women with intra-amniotic infection. This finding is consistent with our recent study showing that patients with spontaneous labor at term (i.e. physiological inflammation<sup>19,31,163-167</sup>) had higher amniotic fluid concentrations of gasdermin D compared to those who delivered at term without labor<sup>168</sup>. In the context of preterm labor with sterile intra-amniotic inflammation, gasdermin D could be released by amniotic fluid leukocytes, keratinocytes/epithelial cells, or other cellular components of amniotic fluid as well as by the chorioamniotic membranes and decidual stromal cells and leukocytes. This concept is supported by the fact that alarmins can initiate inflammasome-mediated inflammatory responses in both immune<sup>169-174</sup> and non-immune cells<sup>173,175</sup>, and treatment of chorioamniotic membranes explants with the alarmin HMGB1 upregulates the expression of inflammasome components and released products<sup>43</sup>. In addition, ultrasound-guided intra-amniotic administration of the alarmin S100B induces activation of the inflammasome in the murine fetal membranes prior to preterm birth<sup>40</sup>. Along with clinical studies showing that there is inflammasome activation in the chorioamniotic membranes and amniotic fluid of women with preterm labor and sterile intra-amniotic inflammation<sup>46</sup>, these data suggest that alarmins can induce inflammasome-mediated pyroptosis in patients with spontaneous preterm labor and birth.

An important observation that requires further study is that decidual cells and leukocytes, mostly macrophages and neutrophils, can undergo pyroptosis in women with preterm labor and birth. It would be interesting to investigate whether decidual cells

express differential amounts of active caspase-1 and mature IL-1 $\beta$  in different clinical scenarios: intra-amniotic infection vs. sterile intra-amniotic inflammation.

## Conclusion

The data presented herein provide evidence that the effector molecule of pyroptosis, gasdermin D, can be detected in the amniotic fluid and chorioamniotic membranes of patients with spontaneous preterm labor and either intra-amniotic infection or sterile intra-amniotic inflammation. Moreover, amniotic fluid gasdermin D concentrations in patients with intra-amniotic infection are greater than in those with sterile intra-amniotic inflammation. These findings suggest that pyroptosis driven by either microbes or alarmins is a central pathway associated with pathological intra-amniotic inflammatory responses in patients with spontaneous preterm labor and birth. The current study provides insights into the immune mechanisms underlying the human syndrome of preterm labor. Further mechanistic studies are required to establish whether gasdermin D-mediated pyroptosis is implicated in the pathophysiology of inflammation-associated preterm labor and birth.

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## FIGURE LEGENDS

**Figure 1. Amniotic fluid gasdermin D concentrations in women with spontaneous preterm labor.** Extracellular gasdermin D (ng/mL) was measured in amniotic fluid of women with spontaneous preterm labor who delivered at term (n = 32) and those who delivered preterm without intra-amniotic inflammation (n = 41), with sterile intra-amniotic inflammation (n = 32), or with intra-amniotic infection (n = 19). Data are shown as box-plots, with boxes representing the interquartile range, and midlines representing the median. Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range. Outliers are shown as individual dots.

**Figure 2. Gasdermin D expression in the chorioamniotic membranes of women who underwent spontaneous preterm labor without intra-amniotic inflammation.**

Representative multiplex immunofluorescence images showing the brightfield view, cell segmentation map, nuclear staining (4',6-diamidino-2-phenylindole, DAPI, blue), and protein expression of gasdermin D (green), caspase-1 (yellow), and IL-1 $\beta$  (red) in the chorioamniotic membranes from women who underwent preterm labor and delivered at term (upper rows, left merged image) or preterm (bottom rows, right merged image) without intra-amniotic inflammation. Merged images show the co-localization of gasdermin D, caspase-1, and IL-1 $\beta$  expression. Images taken at 200X magnification. Scale bars = 100  $\mu$ m.

**Figure 3. Gasdermin D expression in the chorioamniotic membranes of women with spontaneous preterm labor and sterile intra-amniotic inflammation or intra-amniotic infection.**

Representative multiplex immunofluorescence images showing the brightfield view, cell segmentation map, nuclear staining (4',6-diamidino-2-phenylindole, DAPI, blue), and protein expression of gasdermin D (green), caspase-1 (yellow), and IL-1 $\beta$  (red) in the chorioamniotic membranes from women who underwent preterm labor and birth with sterile intra-amniotic inflammation (upper rows, left merged image) or intra-amniotic infection (bottom rows, right merged image). Merged images show the co-localization of gasdermin D, caspase-1, and IL-1 $\beta$  expression. Images taken at 200X magnification. Scale bars = 100  $\mu$ m.

Figure 4. Expression of active caspase-1 in decidual cells from women with preterm labor. (A) Representative flow cytometry gating strategy showing the expression of active caspase-1 (FLICA) in non-leukocytes (CD45<sup>-</sup> cells) and leukocytes (CD45<sup>+</sup> cells) isolated from the decidua of women with preterm labor. (B) Representative flow cytometry plots showing the expression of active caspase-1 (FLICA) in macrophages (CD45<sup>+</sup>CD14<sup>+</sup> cells) and neutrophils (CD45<sup>+</sup>CD15<sup>+</sup> cells) in the decidual tissues from women with preterm labor. Red quadrants indicate pyroptotic cells (active caspase-1<sup>+</sup> and permeable cell membrane, DAPI<sup>+</sup>). N=5.

**Table 1.** Clinical and demographic characteristics of the study population

	Patients with preterm labor who delivered at term (n = 32)	Patients with preterm labor who delivered preterm			P-value
		Without intra-amniotic inflammation or infection (n = 41)	With sterile intra-amniotic inflammation (n = 32)	With intra-amniotic infection (n = 19)	
Maternal age (years; median [IQR]) <sup>a</sup>	23 (20.8-25)	23 (20-26)	24 (20-28)	23 (20-26)	0.6
Body mass index (kg/m <sup>2</sup> ; median [IQR]) <sup>a</sup>	21.6 (19.8-29.5) <sup>c</sup>	24.2 (20.8-28.8) <sup>c</sup>	27.5 (23-33.3) <sup>c</sup>	24.4 (21.5-32.8) <sup>c</sup>	0.1
Primiparity <sup>b</sup>	18.8% (6/32)	29.3% (12/41)	31.3% (10/32)	21.1% (4/19)	0.6
Race/ethnicity <sup>b</sup>					0.8
Black	96.9% (31/32)	85.4% (35/41)	87.5% (28/32)	94.7% (18/19)	
White	0% (0/32)	7.3% (3/41)	6.3% (2/32)	5.3% (1/19)	
Hispanic	0% (0/32)	4.9% (2/41)	3.1% (1/32)	0% (0/19)	
Other	3.1% (1/32)	2.4% (1/41)	3.1% (1/32)	0% (0/19)	
Gestational age at amniocentesis (weeks; median [IQR]) <sup>a</sup>	31.4 (30.4-32.9)	31.4 (27-32.7)	25.2 (23.3-29.9)	28 (23.1-31.5)	<0.001
Gestational age at delivery (weeks; median [IQR]) <sup>a</sup>	38.7 (37.4-39.4)	34.3 (32.1-36)	26.1 (24.3-31.1)	28.1 (23.1-32.4)	<0.001
Birthweight (g) <sup>a</sup>	3072.5 (2900-3388.8)	2260 (1612.5-2491.3) <sup>c</sup>	849 (565-1530)	1120 (560.5-1980)	<0.001
Apgar score at 1 min <sup>a</sup>	9 (8-9)	8 (7-9) <sup>c</sup>	5.5 (2-8)	4 (1.5-7.5)	<0.001
Apgar score at 5 min <sup>a</sup>	9 (9-9)	9 (8-9) <sup>c</sup>	7 (5.8-9)	7 (2.5-8)	<0.001
Acute histologic chorioamnionitis <sup>b</sup>					
Stage 1 (acute subchorionitis)	13.3% (4/30) <sup>c</sup>	11.1% (4/36) <sup>c</sup>	24.1% (7/29) <sup>c</sup>	11.1% (2/18) <sup>c</sup>	0.5
Stage 2 (acute chorioamnionitis)	16.7% (5/30) <sup>c</sup>	19.4% (7/36) <sup>c</sup>	13.8% (4/29) <sup>c</sup>	22.2% (4/18) <sup>c</sup>	0.8

Stage 3 (acute necrotizing chorioamnionitis)	0% (0/30) <sup>c</sup>	2.8% (1/36) <sup>c</sup>	27.6% (8/29) <sup>c</sup>	61.1% (11/18) <sup>c</sup>	<0.001
Acute funisitis <sup>b</sup>					
Stage 1 (acute phlebitis/chronic vasculitis)	13.3% (4/30) <sup>c</sup>	11.1% (4/36) <sup>c</sup>	20.7% (6/29) <sup>c</sup>	27.8% (5/18) <sup>c</sup>	0.3
Stage 2 (acute arteritis)	3.3% (1/30) <sup>c</sup>	2.8% (1/36) <sup>c</sup>	6.9% (2/29) <sup>c</sup>	44.4% (8/18) <sup>c</sup>	<0.001
Stage 3 (necrotizing funisitis)	0% (0/30) <sup>c</sup>	2.8% (1/36) <sup>c</sup>	3.5% (1/29) <sup>c</sup>	0% (0/18) <sup>c</sup>	0.8

Data are given as median (interquartile range) and percentage (n/N)

<sup>a</sup>Kruskal-Wallis test

<sup>b</sup>Fisher's exact test

<sup>c</sup>Few missing data

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**Table 2.** Amniotic fluid gasdermin D concentrations above or below the limit of detection (0.249 ng/mL) in the study groups.

Gasdermin concentration	Preterm labor who delivered at term	Preterm labor who delivered preterm		
		Without intra-amniotic inflammation	With sterile intra-amniotic inflammation	With intra-amniotic infection
>0.249 ng/mL	3.1% (1/32)	4.9% (2/41)	50% (16/32)	89.5% (17/19)
<0.249 ng/mL	96.9% (31/32)	95.1% (39/41)	50% (16/32)	10.5% (2/19)

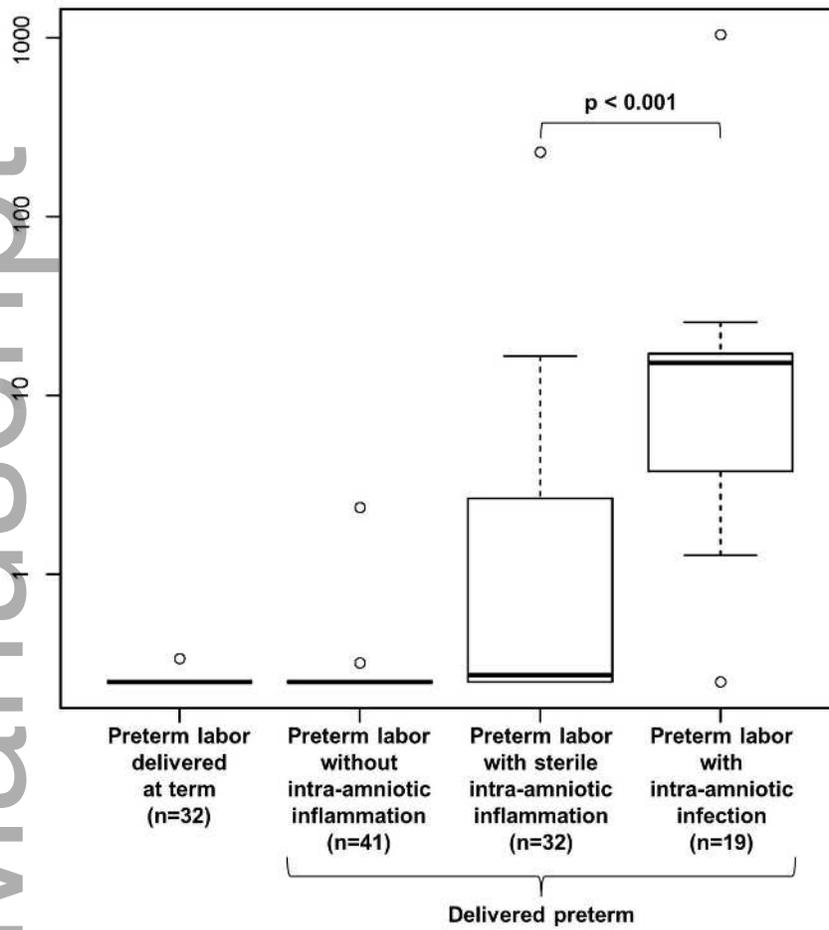
Data are given as percentage (n/N)

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**Table 3.** Association between the frequency of amniotic fluid gasdermin D concentrations > 0.249 ng/mL and the presence of sterile intra-amniotic inflammation or intra-amniotic infection in women who underwent spontaneous preterm labor

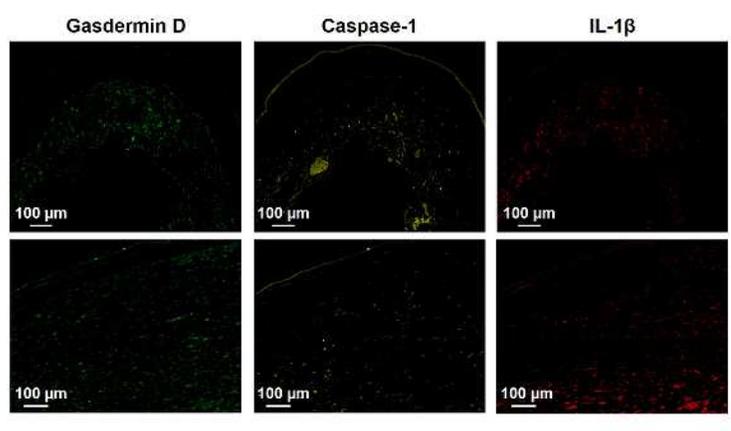
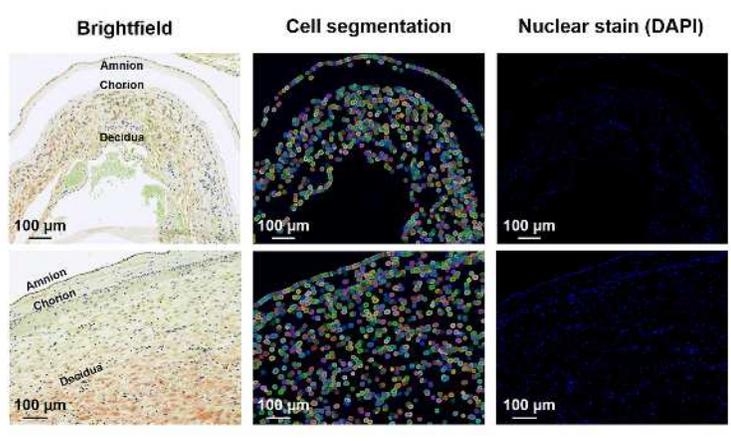
Group comparisons	Relative Risk	P value
Preterm labor who delivered preterm without intra-amniotic inflammation vs. Preterm labor who delivered at term	1.2 (0.52-2.74)	0.7
Preterm labor who delivered preterm with sterile intra-amniotic inflammation vs. Preterm labor who delivered at term	2.76 (1.83-4.19)	<0.001
Preterm labor who delivered preterm with intra-amniotic infection vs. Preterm labor who delivered at term	15.58 (4.05-59.99)	<0.001
Preterm labor who delivered preterm with sterile intra-amniotic inflammation vs. Preterm labor who delivered preterm without intra-amniotic inflammation	3.06 (1.96-4.76)	<0.001
Preterm labor who delivered preterm with intra-amniotic infection vs. Preterm labor who delivered preterm without intra-amniotic inflammation	18.34 (4.71-71.5)	<0.001
Preterm labor who delivered preterm with intra-amniotic infection vs. Preterm labor who delivered preterm with sterile intra-amniotic inflammation	4.64 (1.2-17.85)	0.004

Relative risks are shown with 95% confidence intervals. P-values were obtained using the Chi-square test.

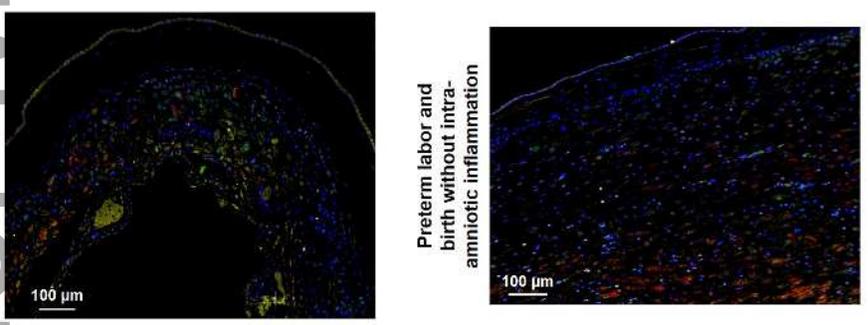


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Preterm labor and birth with term delivery  
Preterm labor and birth without intra-amniotic inflammation  
Preterm labor and birth with term delivery  
Preterm labor and birth without intra-amniotic inflammation

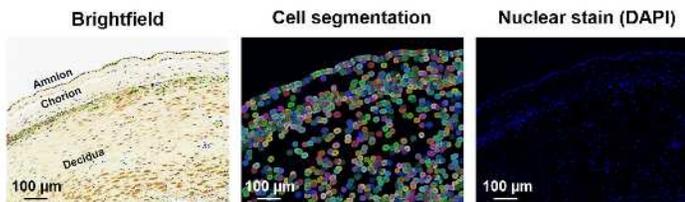


Merged fluorescence images

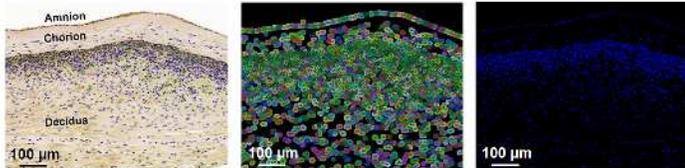


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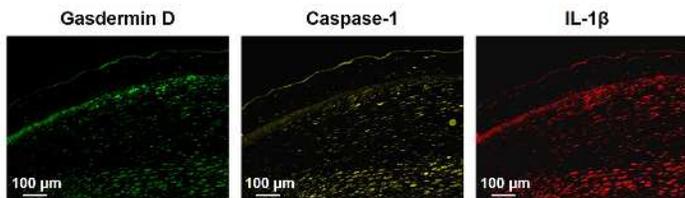
Preterm labor and birth with sterile intra-amniotic inflammation



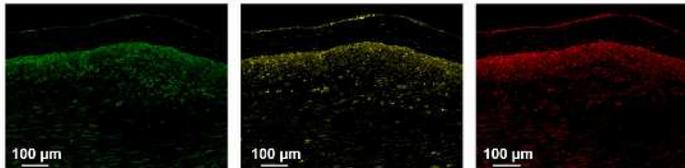
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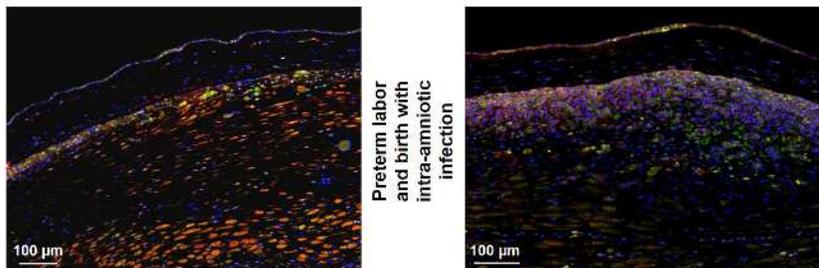
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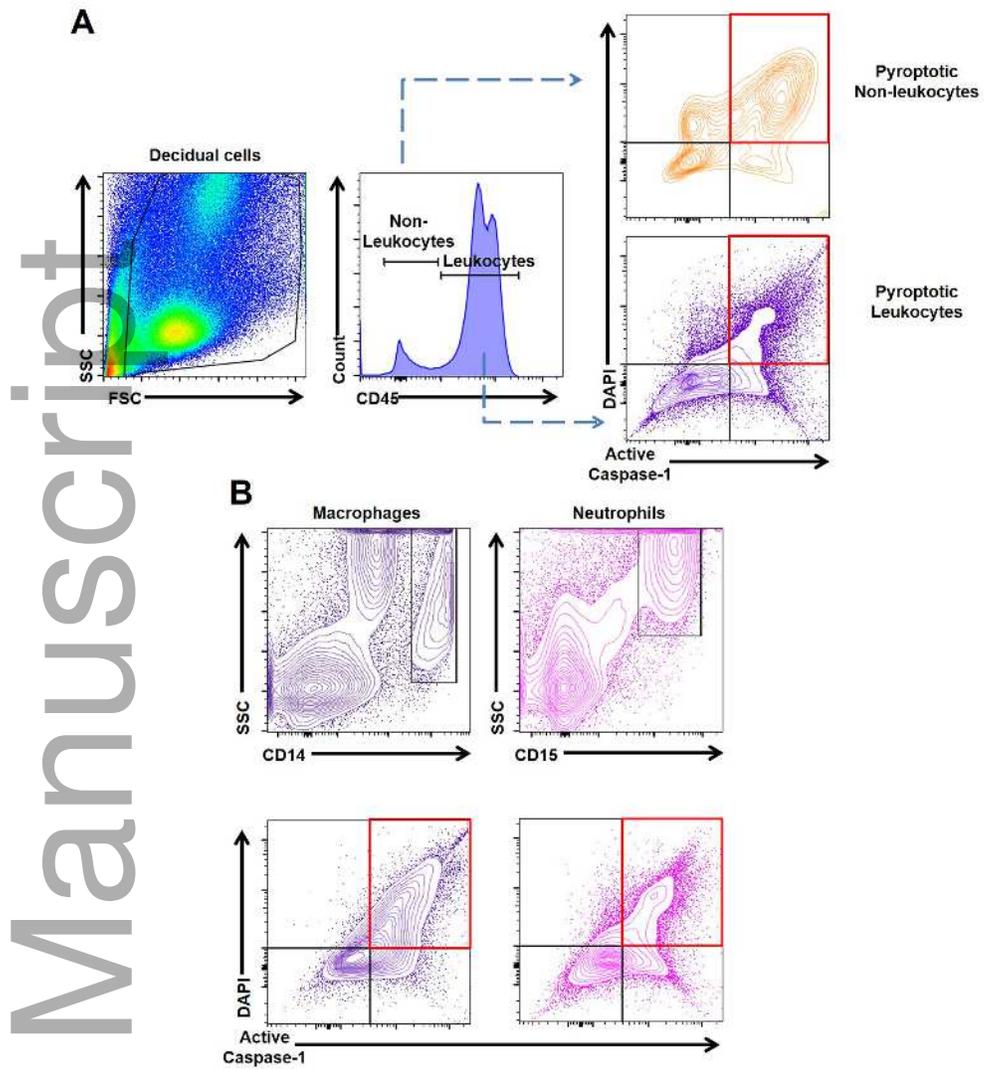
Preterm labor and birth with intra-amniotic infection



Merged fluorescence images



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