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The Immunophenotype of Amniotic Fluid Leukocytes in Normal and Complicated Pregnancies

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ABSTRACT

Problem: The immune cellular composition of amniotic fluid is poorly understood. Herein, we determined the immunophenotype of amniotic fluid: 1) immune cells during the second and third trimester; 2) T cells and innate lymphoid cells (ILCs); and 3) immune cells during intra-amniotic infection/inflammation.

Method of Study: Amniotic fluid samples (n=57) were collected from women from 15-40 weeks of gestation without intra-amniotic infection/inflammation. Samples from women with intra-amniotic infection/inflammation were also included (n=9). Peripheral blood mononuclear cells from healthy adults were used as controls (n=3). Immunophenotyping was performed using flow cytometry.
Results: In the absence of intra-amniotic infection/inflammation, the amniotic fluid contained several immune cell populations from 15-40 weeks. Among these immune cells: 1) T cells and ILCs were greater than B cells and NK cells between 15 to 30 weeks; 2) T cells were most abundant between 15 to 30 weeks; 3) ILCs were most abundant between 15 to 20 weeks; 4) B cells were scarce between 15 to 20 weeks; yet, they increased and were constant after 20 weeks; 5) NK cells were greater between 15 to 30 weeks than at term; 6) ILCs expressed high levels of RORγt, CD161, and CD103 (i.e. Group 3 ILCs); 7) T cells expressed high levels of RORγt; 8) neutrophils increased as gestation progressed; and 9) monocytes/macrophages emerged after 20 weeks and remained constant until term. All of the amniotic fluid immune cells, except ILCs, were increased in the presence of intra-amniotic infection/inflammation.
Conclusions: The amniotic fluid harbors a diverse immune cellular composition during normal and complicated pregnancies.**KEYWORDS:** Immune cells, Leukocytes, Fetal Immunity, Bacteria, Microbes, Mucosal Immunity, T cells, B cells, Innate Lymphoid Cells, NK cells, Neutrophils, Monocytes, Macrophages, Microbial Invasion of the Amniotic Cavity, Intra-amniotic infection, Intra-amniotic inflammation

INTRODUCTION

Amniotic fluid is the protective liquid that surrounds the fetus throughout gestation, and therefore is essential for fetal development and maturation[1]. Besides containing nutrients and other factors required for fetal growth, the amniotic fluid provides mechanical cushioning and represents an immunological barrier against invading pathogens[1, 2]. The amniotic fluid is used as a diagnostic tool for assessing fetal well-being[3-7], lung maturity[8-11], karyotype[12-15] and intra-amniotic infection and/or inflammation[16-74]. During early development, the amniotic fluid is an extension of the fetal extracellular matrix[75]. As the placenta and fetal vessels emerge, water and solutes from the maternal plasma diffuse into the amniotic fluid[76]. By 8 weeks of gestation, the urethra is formed and the fetal kidneys start producing urine[77]. Shortly after, fetal

swallowing begins[75]; however, these processes do not contribute to the amniotic fluid volume until the second half of pregnancy[77]. After 25 weeks of gestation, the fetal skin is fully keratinized[78] and the amniotic fluid volume is determined by factors comprising the amniotic fluid circulation (fetal urine, respiratory system, gastrointestinal system, umbilical cord, and placenta)[75, 77-80].

The amniotic fluid contains soluble and cellular components[77]. The soluble components include carbohydrates, proteins, peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones, among others[81-99], many of which act as the first line of defense against pathogens invading the amniotic cavity[100-106]. The cellular components of the amniotic fluid include different cell types derived from exfoliating surfaces of the developing fetus, including the skin, respiratory system, urinary tract, and gastrointestinal tract, as well as stem cells[107-115]. Some cytological studies have shown that, in the absence of infection, the amniotic fluid also includes a low number of innate immune cells including macrophages[110, 111, 116-118], neutrophils[23, 69], and the recently described innate lymphoid cells or ILCs[119]. Yet, the number of macrophages and/or ILCs is increased in pathological conditions in which fetal organs are exposed to the amniotic fluid (e.g. neural tube defects[118, 120-125] and gastroschisis[118, 126-128]). The number of amniotic fluid neutrophils, on the other hand, is a useful marker for intra-amniotic inflammation[23, 27, 67-69, 129-132]. However, in the absence of infection and/or inflammation or birth defects, the immune cellular composition of the amniotic fluid is still poorly understood.

The aims of this study were: 1) to determine the immunophenotype of amniotic fluid leukocytes during the second and third trimester, 2) to investigate whether amniotic fluid T cells and ILCs display different phenotypical characteristics to that of peripheral cells; and 3) to evaluate whether the amniotic fluid immune cells are altered in women with intra-amniotic infection/inflammation.

MATERIALS AND METHODS

Study population

This was a cross-sectional study of patients who underwent transabdominal amniocentesis due to clinical indications or amniocentesis during cesarean section. Patients were enrolled at Hutzel Women's Hospital of the Detroit Medical Center (September 2016 – January 2017). The first group of patients (n=57, absence of intra-amniotic infection/inflammation) was selected based on the following exclusion criteria: positive amniotic fluid culture[20, 39, 54], white blood cell (WBC) count >50 cells/mm^3 [23], Glucose concentration <14 mg/dL[22], amniotic fluid interleukin (IL)-6 concentration >2.6 ng/mL[39], positive Gram stain[17] and/or bacterial live/dead staining[68], and samples from women with an intra-uterine fetal demise and/or birth defects. A second group of patients with intra-amniotic infection/inflammation was also included (n=9). Intra-amniotic infection/inflammation was defined as the presence of microbial invasion of the amniotic cavity (MIAC) with intra-amniotic inflammation[39, 47, 48, 50-56]. All of the samples with visible blood contamination were excluded from this study. Viable cell numbers were determined using an automatic cell counter (Cellometer Auto 2000, Nexcelom Bioscience, Lawrence, MA, USA).

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

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[23, 54]. Shortly after collection, WBC count in amniotic fluid samples was determined by using a hemocytometer chamber, according to methods previously described[23]. Glucose concentration[22] was also determined and Gram stain[17] was performed in amniotic fluid samples. Cultures, WBC count, glucose concentration, and Gram Stain were not performed in amniotic fluid samples collected during cesarean

section, since these samples were collected for research purposes only. However, both IL-6 concentration[39] and the presence of bacteria (bacterial live/dead staining[68]) were assessed in all of the amniotic fluid samples.

Determination of interleukin-6 in the amniotic fluid

IL-6 concentrations in the amniotic fluid were determined using a sensitive and specific enzyme immunoassay obtained from R&D systems (Minneapolis, MN, USA). The IL-6 concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL.

Detection of live/dead bacteria in the amniotic fluid

The presence of bacteria in the amniotic fluid (n=66) was evaluated as previously described[68, 133], using the LIVE/DEAD BacLight™ Bacterial Viability Kit (Cat# L7007, Life Technologies, Grand Island, New York) in a sterile biosafety cabinet. Briefly, 100μL of amniotic fluid were mixed with 900μL of sterile 1X phosphate buffered saline (PBS; Life Technologies). Three microliters of the dye mix (Component A and B were mixed at a 1:1 ratio) were added to the cell suspension and incubated for 15 min at room temperature in the dark. Next, the cells were centrifuged at 10,000 x g for 5 min and the supernatant was discarded. The cell pellet was then re-suspended in 5μL of 1X PBS, and a slide smear was prepared and air-dried. Lastly, the slide was gently rinsed with 1X PBS and mounted with ProLong Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole or DAPI (Life Technologies). The presence of bacteria was evaluated using an Olympus BX 60 fluorescence microscope with an Olympus DP71 camera and DP Controller Software (Olympus Corporation, Tokyo, Japan).

Isolation of peripheral blood mononuclear cells

Peripheral blood samples were collected by venipuncture into EDTA-containing tubes from healthy individuals (n=3). Peripheral blood mononuclear cells (PBMCs) were isolated using the density gradient reagent Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions.

Immunophenotyping

Amniotic fluid samples (5 to 6 mL; n=66) were passed through a sterile15-µm filter to remove most epithelial cells and centrifuged at 200 x g for 5 minutes at room temperature. The resulting amniotic fluid leukocyte pellet or PBMCs were re-suspended in 1mL of 1X PBS and stained with the BD Horizon Fixable Viability Stain 510 dye (BD Biosciences, San Jose, CA, USA), prior to incubation with extracellular monoclonal antibodies (Supplementary Table 1). Cells were washed in 1X PBS and incubated with 20µL of human FcR blocking reagent (Miltenyi Biotec, San Diego, CA, USA) in 80µL of BD FACS stain buffer for 10 min at 4℃. Next, cells were incubated with extracellular fluorochrome-conjugated anti-human monoclonal antibodies for 30 min at 4℃ in the dark (Supplementary Table 1). Following extracellular staining, cells were fixed and permeabilized using the FoxP3 Transcription Factor Fixation/Permeabilization solution (Cat#00-5523-00; eBioscience, San Diego, CA, USA). Following fixation and permeabilization, cells were washed with 1X FoxP3 Permeabilization Buffer (eBioscience), re-suspended in 50 µl of the same buffer, and stained with intracellular antibodies for 30 minutes at 4℃ in the dark (Supplementary Table 2). Isotope controls were also prepared. Stained cells were then washed with 1X permeabilization buffer, re-suspended in 0.5mL of BD FACS stain buffer, and acquired using the BD LSR Fortessa Flow Cytometer (BD Bioscience) and BD FACSDiva 6.0 software (BD Bioscience). The analysis was performed and the figures were generated using the FlowJo v10 software (FlowJo, Ashland, OR, USA). The absolute number of cells was determined using CountBright absolute counting beads (Molecular Probes, Eugene, OR, USA).

Imaging flow cytometry of amniotic fluid leukocytes

Amniotic fluid samples (1-10 mL; n=5) were passed through a sterile15-µm filter (Cat# 43-50015-03; pluriSelect Life Science, Leipzig, Germany) to remove most epithelial cells and centrifuged at 200 x g for 5 minutes at room temperature. The cell pellet (mostly leukocytes) was washed with 1X PBS, re-suspended in 80 µL of BD FACS stain buffer (Cat#554656; BD Biosciences) containing 20 µL of human FcR blocking reagent (Miltenyi Biotec), and incubated for 10 min at 4℃. Next, the amniotic fluid leukocytes were stained separately with the following two panels of extracellular fluorochrome-conjugated anti-human antibodies (BD Biosciences) for 30 min at 4℃ in

the dark. One antibody panel included CD45-APC (Clone HI30, Cat#555485), CD56-PE (clone NCAM16.2, Cat#340363), CD14- Alexa Fluor® 488 (clone MφP9, Cat#562689) and CD15-PE-CF594 (clone W6D3, Cat#562372). The second panel included CD45-APC (Clone HI30), CD19- PE (clone HIB19, Cat#555413), CD3- Alexa Fluor® 488 (clone UCHT1, Cat#557694) and CD127-PE-CF594 (clone HIL-7R-M21, Cat#562397). Following antibody staining, amniotic fluid leukocytes were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 20 min at room temperature. Amniotic fluid cells were then rehydrated in 1x PBS and stained with 3 μM DAPI (Cat#D9542, Sigma, St. Louis, MO, USA) in a nuclear permeabilization buffer (Cat#00-8333-56, eBiosciences) for 15 min at room temperature. Lastly, amniotic fluid cells were suspended in 50 μL BD FACS stain buffer containing 1mg/ml EDTA (Cat#15575-038, Life Technologies). Samples were acquired using an ImageStream®^{X} Mk II imaging cytometer (Amnis, Seattle, WA) at the Microscopy, Imaging, and Cytometry Resources Core at the Wayne State University School of Medicine (Detroit, MI, USA) (http://micr.med.wayne.edu/). Images were obtained at a magnification of 60X using the low flow rate/high sensitivity INSPIRE software (Amnis) and the following lasers: 405nm, 488nm and 642nm. Acquired images were analyzed using the IDEALS 6.2 software (Amnis).

Statistical Analysis

Statistical analysis was performed using SPSS v.19.0 software (SPSS Inc., IBM Corporation, Armonk, NY). Kruskal–Wallis tests followed by Mann-Whitney U-tests were used for unpaired comparisons among study groups. Wilcoxon signed-rank tests were used for paired comparisons. t-SNE plots were generated using the FlowJo v10 software. A p-value ≤ 0.05 was considered statistically significant.**RESULTS**

Characteristics of the study population

A total of 66 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 Tables 1 and 2. Most of the amniotic fluid samples were collected from African-American women (Table 1). The number of amniotic fluid samples per group is displayed in Table 2. Amniotic fluid samples obtained between 15 and 20 weeks of gestation were mostly

collected from women who underwent an amniocentesis for fetal karyotyping and delivered term neonates (Table 2). Amniotic fluid samples obtained between 20-36 weeks of gestation were collected from women who underwent an amniocentesis for the detection of intra-amniotic infection and/or inflammation (negative) and delivered preterm neonates (Table 2). Amniotic fluid samples obtained between 36-40 weeks of gestation were mostly collected from women during the cesarean section procedure and who delivered term neonates (Table 2). Amniotic fluid samples from women with intra-amniotic infection/inflammation were collected from 18 to 40 weeks of gestation (Table 2).

The immunophenotype of amniotic fluid leukocytes during second and third trimester in the absence of intra-amniotic infection/inflammation

First, we investigated the diversity of amniotic fluid leukocytes during the second and third trimesters. Figure 1 shows representative t-SNE plots demonstrating that different immune cell populations are present throughout gestation. T cells and ILCs were the most abundant immune cell populations between 15 to 20 weeks of gestation (Figure 1A). Between 20 to 30 weeks of gestation, B cells, monocytes and neutrophils emerged, and while T cells were still abundant ILCs were drastically reduced (Figure 1B). Between 30 to 36 weeks of gestation, neutrophils and monocytes were greater than other cell types, and B cells were lower than earlier in gestation (Figure 1C). Lastly, neutrophils were the larger immune cell population between 37-40 weeks of gestation; yet, monocytes were still abundant and B cells were detectable (Figure 1D).

Lymphoid cells in the amniotic fluid during the second and third trimester in the absence of intra-amniotic infection/inflammation

Lymphoid cells were abundant between 15 to 30 weeks of gestation (Figure 1A&B); therefore, we first determined which lymphoid subset was more abundant during this period. The gating strategy used to identify lymphoid cells in the amniotic fluid is shown in Figure 2A. Briefly, total leukocytes were identified as CD45+ cells within the singlets and viability gates. Identified lymphoid cells in the amniotic fluid included: T cells (CD45+CD15-CD14- CD19-CD3+ cells), B cells (CD45+CD15-CD14-CD3-CD19+ cells), NK cells (CD45+CD15-CD14- CD19-CD3-CD19-CD94+CD56+ cells), and ILCs

(CD45+CD15-CD14-CD19-CD3-CD94-CD127+ cells) (Figure 2A). A previous report showed that most of the amniotic fluid ILCs expressed CD56[119]; therefore, we detected both CD56+ and CD56- ILCs (Figure 2A). Flow cytometry quantification revealed that T cells and ILCs were more abundant than B cells and NK cells in the amniotic fluid of women between 15 to 30 weeks of gestation (Figure 2B). In addition, we found that the CD56+ and CD56- ILC subsets were present in similar numbers (Figure 2C). These data indicate that T cells and ILCs are the most abundant population in the amniotic fluid of women between 15 to 30 weeks of gestation in the absence of intra-amniotic infection/inflammation.

Next, we determined the abundance of all of the lymphoid cell populations during the second and third trimester (Figure 3). Amniotic fluid T cells were more abundant between 20 to 30 weeks of gestation than after 37 weeks of gestation (Figure 3A). A flow cytometry image of an amniotic fluid T cell during the second trimester is shown in Figure 3B. Amniotic fluid ILCs were most abundant between 15 to 20 weeks of gestation (Figure 3C), as previously reported[119]. Yet, their number reduced after 20 and 30 weeks of gestation and remained low after 37 weeks of gestation (Figure 3C). A flow cytometry image of an amniotic fluid ILC during the second trimester is shown in Figure 3D. Amniotic fluid B cells did not significantly change during the second and third trimester; yet, their lowest number was observed between 15 to 20 weeks of gestation (Figure 3E). A flow cytometry image of an amniotic fluid B cell during the second trimester is shown in Figure 3F. Amniotic fluid NK cells were more abundant between 15 to 30 weeks of gestation than at term (>37 weeks) (Figure 3G). A flow cytometry image of an amniotic fluid NK cell during the second trimester is shown in Figure 3H. Together, these data indicate that amniotic fluid T cells and ILCs, followed by NK cells, are most abundant between 15 to 30 weeks of gestation. These results also show that although B cells are low in number, such cells are a constant immune cell population in the amniotic fluid during the second and trimester in the absence of intra-amniotic infection/inflammation.

Immunophenotyping of ILCs and T cells in the amniotic fluid in the absence of intra-amniotic infection/inflammation

A previous study reported that most of the amniotic fluid ILCs belong to the Group 3 since they express RORγt and CD161 as well as produce high levels of IL-17 and TNFα[119]. It was also shown that these fetal Group 3 ILCs express CD103 (a hallmark of intraepithelial cells) and were present in the amniotic fluid but not in the peripheral circulation[119]. We therefore investigated whether ILCs express RORγt, CD161, and CD103 in the amniotic fluid and peripheral blood of healthy adults (Figure 4A). Most of the amniotic fluid ILCs expressed RORγt as well as were double positive for CD161 and CD103 (i.e. Group 3 ILCs) (Figure 4 B&C); however, the expression of these markers was minimal in peripheral ILCs (Figure 4B&C). Flow cytometric quantification consistently revealed that the proportions of CD56+/CD56-RORγt+ ILCs (Figure 4D&E) and CD56+/CD56-CD161+CD103+ ILCs (Figure 4F&G) were higher in the amniotic fluid than in the peripheral circulation.

T cells were abundant in the amniotic fluid of women between 15 to 30 weeks of gestation; therefore, we determined whether such cells expressed the same markers as ILCs. Similar to amniotic fluid ILCs, a high proportion of amniotic fluid T cells (Figure 5A) expressed RORγt (Figure 5B). The proportion of RORγt+ T cells was greater in the amniotic fluid than in the peripheral circulation (Figure 5C). Distinct to amniotic fluid ILCs, only ~30% of amniotic fluid T cells were double positive for CD161 and CD103 (Figure 5D). Yet, the proportion of CD161+CD103+ T cells was higher in the amniotic fluid than in the peripheral circulation (Figure 5E).

Taken together, these results indicate that Group 3 ILCs and RORγt+ T cells are present in the amniotic fluid between 15 to 30 weeks of gestation in the absence of intra-amniotic infection/inflammation.

Myeloid cells in the amniotic fluid during the second and third trimester in the absence of intra-amniotic infection/inflammation

Next, we quantified the number of myeloid cells in the amniotic fluid of women during the second and third trimester. The gating strategy used to identify neutrophils and monocytes in the amniotic fluid is shown in Figure 6A. Briefly, total leukocytes were identified as CD45+ cells within the singlets and viability gates. Identified myeloid cells in the amniotic fluid included neutrophils (CD45+CD14-CD15+ cells) and monocytes/macrophages (CD45+CD15-CD14+ cells) (Figure 6A). The number of

amniotic fluid neutrophils increased gradually from 15 weeks to term gestation (Figure 6B). The number of amniotic fluid monocytes/macrophages was greater between 20 to 40 weeks compared to 15-20 weeks of gestation (Figure 6C). Yet, the number of these myeloid cells did not peak at term pregnancy as occurred with amniotic fluid neutrophils (Figure 6B*vs.*C). Lastly, neutrophils were more abundant than monocytes/macrophages in the amniotic fluid of women at term (Figure 7A). A flow cytometry image of an amniotic fluid neutrophil or monocyte/macrophage during the third trimester is shown in Figure 7B or 7C, respectively. These data show the number of amniotic fluid neutrophils increases as gestation progresses, and that monocyte/macrophages are a constant immune cell population after 20 weeks of gestation in the absence of intra-amniotic infection/inflammation.

Immune cells in the amniotic fluid of women with intra-amniotic infection/inflammation

Previous studies have shown that intra-amniotic infection is characterized by the infiltration of neutrophils and monocytes/macrophages[23, 27, 67-69, 129-132]. It was recently suggested that amniotic fluid ILCs were also implicated in regulating intra-amniotic infection and inflammation in preterm gestations[119]. Therefore, we evaluated whether amniotic fluid immune cells were increased in women with intra-amniotic infection/inflammation. All of the amniotic fluid immune cells, except ILCs, were increased in cases with intra-amniotic infection/inflammation (Figure 8). The numbers of ILCs in the amniotic fluid collected at 18-22 weeks of gestation from women with intra-amniotic infection/inflammation were similar to those in gestational age-matched amniotic fluids from women without intra-amniotic infection/inflammation (Supplementary Figure 1). The numbers of neutrophils and monocytes/macrophages were greater than T cells, ILCs, B cells, and NK cells in the amniotic fluid of women with intra-amniotic infection/inflammation (Figure 8). Collectively, these data indicate that all of the amniotic fluid immune cells, except ILCs, are implicated in the inflammatory response implicated in intra-amniotic infection/inflammation.

DISCUSSION

Principal findings: In the absence of intra-amniotic infection/inflammation: 1) several immune cell populations were detected in the amniotic fluid during the second and third trimester; 2) T cells and ILCs were greater than B cells and NK cells in the

amniotic fluid between 15 to 30 weeks of gestation; 3) amniotic fluid T cells were most abundant between 15 to 30 weeks of gestation; 4) amniotic fluid ILCs were most abundant between 15 to 20 weeks of gestation; 5) amniotic fluid B cells did not significantly vary during the second and third trimester, yet, they were scarce between 15 to 20 weeks of gestation; 6) amniotic fluid NK cells were greater between 15 to 30 weeks of gestation than at term; 7) amniotic fluid ILCs expressed high levels of RORγt, CD161, and CD103 (i.e. Group 3 ILCs); 8) amniotic fluid T cells expressed high levels of RORγt; 9) amniotic fluid neutrophils increased as gestation progressed; 10) amniotic fluid monocytes/macrophages emerged after 20 weeks of gestation and remained constant until term; and 11) neutrophils were more abundant than monocytes/macrophages in the amniotic fluid of women at term. Lastly, we found that all of the amniotic fluid immune cells, except ILCs, were increased in the presence of intra-amniotic infection/inflammation.

Amniotic fluid innate lymphoid cells (ILCs)

Recently, it was shown that Group 3 ILCs are present in the human amniotic fluid between 15 to 16 weeks of gestation (second trimester)[119]. Such innate lymphocytes expressed the intraepithelial marker CD103, suggesting that they were derived from the fetal intestine[119]. This concept was supported by the fact that Group 3 ILCs were found in the fetal small intestine and lung, and these cells displayed a similar phenotype to that detected in the amniotic fluid[119]. Herein, we extended these findings by demonstrating that the human amniotic fluid contains Group 3 ILCs (RORγt+CD161+CD103+ ILCs) during the second and third trimester; yet, their number is highest earlier in pregnancy.

Previous studies have shown that fetal tissues include different subsets of ILCs. In humans, both ILC2-like (Lin-CRTH2+CD127+ cells) and ILC3-like (Lin-CD127+NKp44+ expressing RORγt) subsets were found in the fetal gut at 14-17 weeks of gestation[134]. In addition, Group 2 ILCs (GATA3+ ILCs) have been detected in the umbilical cord blood of term neonates[135]. In mice, fetal RORγt+ ILC progenitors mature in the fetal liver environment[136, 137] in a Notch2-dependent manner[138]. However, non-hepatic ILC progenitors may exist in the fetal intestine[139]. While Group 3 ILCs exist in the fetal murine intestine early in pregnancy, a particular subset which is only a fraction

of murine intestinal ILCs seems to rapidly expand shortly after birth[140], which may be related to the colonization of the fetal gut by commensal microbes. Moreover, fetuses with gastroschisis had increased Group 2 and Group 3 ILCs in the sections of intestine exposed to the amniotic fluid, suggesting that these cells participate in the inflammatory environment that leads to fetal bowel damage[128].

Herein, we showed that amniotic fluid ILCs were not significantly increased in women with intra-amniotic infection/inflammation between 18 to 22 weeks of gestation; yet, this finding needs to be further investigated using a larger number of samples. Collectively, these studies demonstrate that ILCs, in particular Group 3 ILCs, are an important immune cell subset in both the amniotic cavity and the developing fetus.

Amniotic fluid T cells

To our knowledge, we are the first to report that T cells are an abundant immune cell population in the amniotic fluid between 15 to 30 weeks of gestation (mostly the second trimester). It is likely that these adaptive immune cells are derived from the fetus since most of the amniotic fluid leukocytes in early gestation are of fetal origin[67]. In addition, amniotic fluid T cells expressed high levels of RORγt similar to what was observed in amniotic fluid Group 3 ILCs of fetal origin[119].

In humans, T cells are detected in the fetal lymphoid tissues as early as 10 weeks of gestation[141]. By 12-14 weeks of gestation, T cells are found in the fetal intestine, spleen, and lymph nodes; yet, they are more abundant by the end of the second trimester[142]. At this time, secondary fetal lymphoid tissues contain a high proportion of CD4+CD25+FoxP3+ regulatory T cells or Tregs[143, 144] which are mostly generated in response to maternal alloantigens[145]. Fetal Tregs strongly suppress both natural fetal T-cell activity[144] and responses against maternal antigens to prevent maternal-fetal rejection[143, 144, 146]. The induction of maternal-specific fetal Tregs is orchestrated by transforming growth factor β (TGF-β), which is produced by the fetal lymph nodes[145].

Th17 cells have also been identified in the cord blood of term neonates; yet, their proportions are higher in preterm neonates exposed to histologic chorioamnionitis[147]. Th17 cells are characterized by the expression of RORγt and production of IL-17[148]. Indeed, Tregs can promote a Th17-like phenotype in the context of inflammation[149] and Tregs can express RORγt to enhance their suppressive activity during intestinal

inflammation[150]. Here, we identified T cells in the amniotic fluid that express RORγt, which are greater in cases with intra-amniotic infection/inflammation, suggesting that these cells participate in the inflammatory process against microbial invasion of the amniotic cavity. Further research is required in order to investigate whether amniotic fluid T cells are indeed of fetal origin and whether these cells have pro-inflammatory and/or immunosuppressive functions.

Amniotic fluid B cells

The data herein showed that B cells are constantly present during the second and third trimester; yet, these cells are very rare between 15-20 weeks of gestation. Previous observations[67, 119] make us suggest that amniotic fluid B cells may be of fetal origin. Fetal pro/pre-B cells can be detected in the omentum as early as 8 weeks of gestation and B-cells can be detected in the liver and spleen at 12 weeks[151]. At this point, approximately 40% of these B cells express CD5, a marker of fetal B-cell linage[152, 153]. By 23 weeks of gestation, 90% of fetal splenic B cells express CD5 (B1-like cells), which gradually reduce to adult levels (25-35%) by late adolescence[154]. B-1 cells (CD5+ B cells) are responsible for production of antibodies in response to bacterial cell wall components[155]. Therefore, these cells limit bacterial colonization before induction of adaptive immune responses[156, 157]. Indeed, it was also suggested that the fetal B cell system could be considered an intermediate between the innate immune system and the adaptive immune system[158]. Here, we provided evidence that amniotic fluid B cells are increased in women with intra-amniotic infection/inflammation supporting the hypothesis that these adaptive immune cells participate in the fetal host response against microbial invasion of the amniotic cavity.

Amniotic fluid natural killer (NK) cells

In the current study, we found that the amniotic fluid includes NK cells, which are most abundant between 15 to 30 weeks of gestation (mostly the second trimester). It is likely that amniotic fluid NK cells are derived from the fetus since most of the identified immune cells in this compartment are of fetal origin during this period[67, 119].

Fetal NK cells are detected as early as 6 weeks of gestation[159]. After 18 weeks of gestation, the proportion of NK cells is increased in the fetal liver equaling the proportion of T cells[159]. Fetal NK cells are implicated in cytokine- and antibody-mediated NK cell

responses in utero; yet, they remain hyporesponsive to HLA class I–negative or allogeneic cells[160], which could be considered a mechanism for maternal-fetal tolerance[161, 162]. Taken together, these findings suggest that amniotic fluid NK cells could participate in the mechanisms of maternal-fetal tolerance taking place in the fetal compartments. The fact that amniotic fluid NK cells are increased in women with intra-amniotic infection/inflammation suggests that these cells are also implicated in the fetal host response against microbial invasion of the amniotic cavity.

Amniotic fluid neutrophils

Our results showed that amniotic fluid neutrophils increased as gestation progressed, and their number was even greater in women with intra-amniotic infection/inflammation. Amniotic fluid neutrophils can be of fetal and/or maternal origin[67]. Amniotic fluid neutrophils are mostly of fetal origin during preterm gestation whereas these cells are of maternal origin at term[67]. Regardless of their origin, amniotic fluid neutrophils are a part of the innate immune host defense mechanisms that take place in the amniotic cavity of women with intra-amniotic infection[77, 163, 164]. This concept is supported by evidence demonstrating that amniotic fluid neutrophils 1) are a source of anti-microbial products[90, 165-168] and cytokines[69], 2) can trap and kill bacteria invading the amniotic cavity by forming neutrophil extracellular traps (NETs)[68], and 3) can phagocytize microorganisms commonly found in the lower genital tract, e.g., *Streptococcus agalactiae* (also known as Group B Streptococcus or GBS), *Ureaplasma urealyticum*, *Gardnerella vaginalis*, and *Escherichia coli*[132]. Together, these findings show that even in the absence of microbial invasion, neutrophils are present in the amniotic fluid throughout gestation and ready to participate in the host defense mechanisms taking place in the amniotic cavity.

Amniotic fluid monocytes/macrophages

Macrophages have been previously observed in the amniotic fluid from normal pregnancies using cytological techniques[110, 111, 116, 117]. By using high dimensional flow cytometry, we found that monocytes/macrophages are consistently present in the amniotic fluid of women after 20 weeks of gestation. Monocytes emerged during the second trimester suggesting that they have a fetal origin, as the neutrophils do[67].

Fetal macrophages are observed in the early stages of embryonic development[169]. Such cells originate in the yolk sac, fetal liver, and bone marrow[169-171] and can colonize developing organs to become tissue residents which persist until adulthood[172]. During pregnancy, Hofbauer cells (fetal macrophages) reside in the placental villous tree[173-175], indicating that this can be a potential source for amniotic fluid macrophages. In cases with intra-amniotic infection/inflammation, monocytes/macrophages are abundant and expressed high levels of IL-1α and IL-1β[69]. These cytokines participate in the process of parturition[176-178] and host response to intra-amniotic infection[176, 178-185].

Conclusions

The amniotic fluid harbors a diverse immune cellular composition during the second and third trimester. Between 15 to 20 weeks, ILCs are the most abundant in the amniotic fluid. T cells and ILCs, followed by NK cells, are more abundant between 15 to 30 weeks than at term. B cells are rare between 15 to 20 weeks but they are a constant immune cell population until full term. While neutrophils increase as gestations progresses, monocytes/macrophages emerged after 20 weeks and remained constant until term. All of the amniotic fluid immune cells, except ILCs, are increased in cases with intra-amniotic infection/inflammation. These findings provide insight into the biology of the amniotic fluid leukocytes during normal and complicated pregnancies.

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FIGURE LEGENDS

Figure 1. The immunophenotype of amniotic fluid leukocytes during the second and third trimester. Flow cytometry analysis of immune cells was performed and t-SNE plots were generated from flow cytometry data. Dot plots (upper row) and contour plots (lower row) represent immune cell diversity and abundance in amniotic fluids collected at **(A)** 15-20, **(B)** 20-30, **(C)** 30-36, or **(D)** 37-40 weeks of gestation. Pink = ILCs, purple = NK cells, orange = B cells, green = T cells, red = monocytes/macrophages, and blue = neutrophils.

Figure 2. T cells and ILCs are the dominant immune cell subsets in the amniotic fluid during the second trimester. **(A)** Flow cytometry gating strategy for immunophenotyping of immune cells. Immune cells were initially gated within the viability gate and CD45+ gate followed by lineage gating (CD15, CD14, CD19, CD3, CD94, CD56, CD127). **(B)** Numbers of T cells, ILCs, B cells, and NK cells in the amniotic fluid of women at 15-30 weeks of gestation. **(C)** Numbers of CD56- or CD56+ ILCs in the amniotic fluid of women at 15-30 weeks of gestation. n=14 per group.

Figure 3. Number of lymphoid cells in the amniotic fluid during the second and third trimester. The numbers of T cells **(A)**, ILCs **(C)**, B cells **(E)**, and NK cells **(G)** in the amniotic fluid of women at 15-20, 20-30, 30-36, or 37-40 weeks of gestation. Imaging flow cytometry analysis showing (from Left to Right): BF, bright field imaging, nuclear (DAPI) staining, CD3 **(B),** CD127+ **(D),** CD19+ **(F),** or CD56 **(H)** expression, as well as CD45 expression, and the merged fluorescence image of an amniotic fluid T, ILC, B, or NK cell, respectively. n=6-34 per group.

Figure 4. Amniotic fluid ILCs expressing RORγt, CD161, and CD103. **(A)** Mononuclear cells were isolated from the amniotic fluid of women at 15-30 weeks of gestation or the peripheral blood (PBMCs) from healthy adults. Flow cytometry gating strategy for immunophenotyping of CD127+ ILCs within the lineage negative (Lineage-; CD15-CD14-CD3-CD19-CD56-CD11b-CD94-) and CD56 positive or negative (CD56+/-) gates. **(B)** Flow cytometry gating strategy for immunophenotyping of RORγt+ ILCs within the Lineage-CD56+CD127+ or Lineage-CD56-CD127+ gates. **(C)** Flow cytometry gating strategy for immunophenotyping of CD103+CD161+ ILCs within the Lineage-CD56+CD127+ or Lineage-CD56-CD127+ gates. **(D)** Percentage of CD127+CD56+RORγt+ ILCs in PBMCs or the amniotic fluid. **(E)** Percentage of CD127+CD56- RORγt+ ILCs in PBMCs or the amniotic fluid. **(F)** Percentage of CD127+CD56+CD161+CD103+ ILCs in PBMCs or the amniotic fluid. **(G)** Percentage of CD127+CD56-CD161+CD103+ ILCs in PBMCs or the amniotic fluid. n=3-11 per group.

Figure 5. Amniotic fluid T cells expressing RORγt, CD161, and CD103. **(A)** Mononuclear cells were isolated from the amniotic fluid of women at 15-30 weeks of gestation or the peripheral blood (PBMCs) from healthy adults. Flow cytometry gating strategy for immunophenotyping of CD3+ T cells within the CD45+CD15-CD14- gate. **(B)** Flow cytometry gating strategy for immunophenotyping of RORγt+ T cells within the CD45+CD15-CD14-CD3+ gate. **(C)** Percentage of CD3+RORγt+ T cells in PBMCs or the amniotic fluid. **(D)** Flow cytometry gating strategy for immunophenotyping of CD103+CD161+ T cells within the CD45+CD15-CD14-CD3+ gate. **(E)** Percentage of CD3+CD161+CD103+ T cells in PBMCs or the amniotic fluid. n=3-12 per group.

Figure 6. Myeloid cells in the amniotic fluid during the second and third trimester. **(A)** Flow cytometry gating strategy for immunophenotyping of myeloid cells. Cells were initially gated within the viability gate and CD45+ gate followed by CD15 and CD14 gating. (B) The numbers of neutrophils in the amniotic fluid of women at 15-20, 20-30, 30-36, or 37-40 weeks of gestation. (C) The numbers of monocytes/macrophages in the amniotic fluid of women at 15-20, 20-30, 30-36, or 37-40 weeks of gestation. n=6-34 per group.

Figure 7. Neutrophils are the dominant myeloid subset in the amniotic fluid at term. **(A)** Numbers of CD15+ neutrophils and CD14+ monocytes/macrophages in the amniotic fluid from women at term. **(B)** Imaging flow cytometry analysis showing (from L-R): BF, bright field imaging, nuclear (DAPI) staining, CD15 expression, CD45 expression, and the merged fluorescence image of an amniotic fluid neutrophil. **(C)** Imaging flow cytometry analysis showing (from L-R): BF, bright field imaging, nuclear (DAPI) staining, CD14 expression, CD45 expression, and the merged fluorescence image of an amniotic fluid monocyte/macrophage. n=34 per group.

Figure 8. Immune cells in the amniotic fluid during intra-amniotic infection/inflammation. The numbers of **(A)** T cells, **(B)** ILCs, **(C)** B cells, **(D)** NK cells, **(E)** neutrophils, and **(F)** monocytes/macrophages in the amniotic fluid of women with or without intra-amniotic infection/inflammation. n=9-57 per group.

Table 1: Demographic characteristics of study population

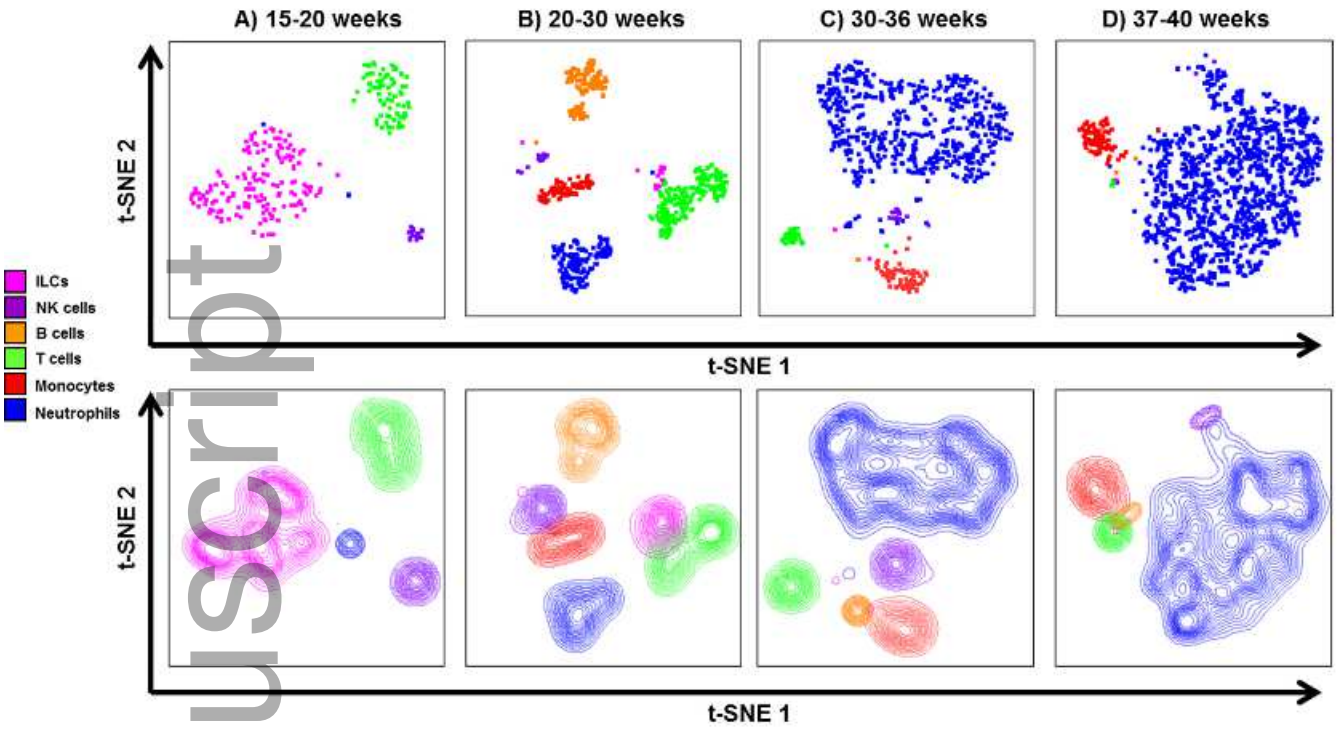
	Patient (n=66)
Maternal Age years; median (IQR)	25.5 (23-29.3)
Body mass index kg/m^2; median (IQR)	30.8 (24.6-38.6)
Primiparity	18.8%
Race	
African-American	89.1%
Caucasian	4.7%
Asian	3.1%
Other	3.1%
Smoking	21.9%

IQR, interquartile range

Table 2. Clinical characteristics of the study population

Groups		Number of samples	Gestational age at amniocentesis weeks; median (IQR)	IL-6 concentration ng/mL; median (IQR)	Gestational age at delivery weeks; median (IQR)
Absence of intra-amniotic infection/ inflammation	**15-20 weeks**	6	17.7 (16.4-18.8)	0.3 (0.1-0.8)	38.4 (38.4-39.3)
	20-30 weeks	8	23.2 (21.6-27.2)	0.5 (0.2-0.9)	28.6 (24.5-35.6)
	30-36 weeks	9	32.3 (31.4-33.3)	0.3 (0.2-0.4)	33.9 (32.3-36)
	37-40 weeks	34	39 (38.9-39.3)	0.3 (0.3-0.7)	39 (38.9-39.3)
Intra-amniotic infection/ inflammation (18-40 weeks)		9	38.1 (22.3-39.6)	70.6 (6.4-118.7)	38.1 (22.7-39.6)

IQR, interquartile range



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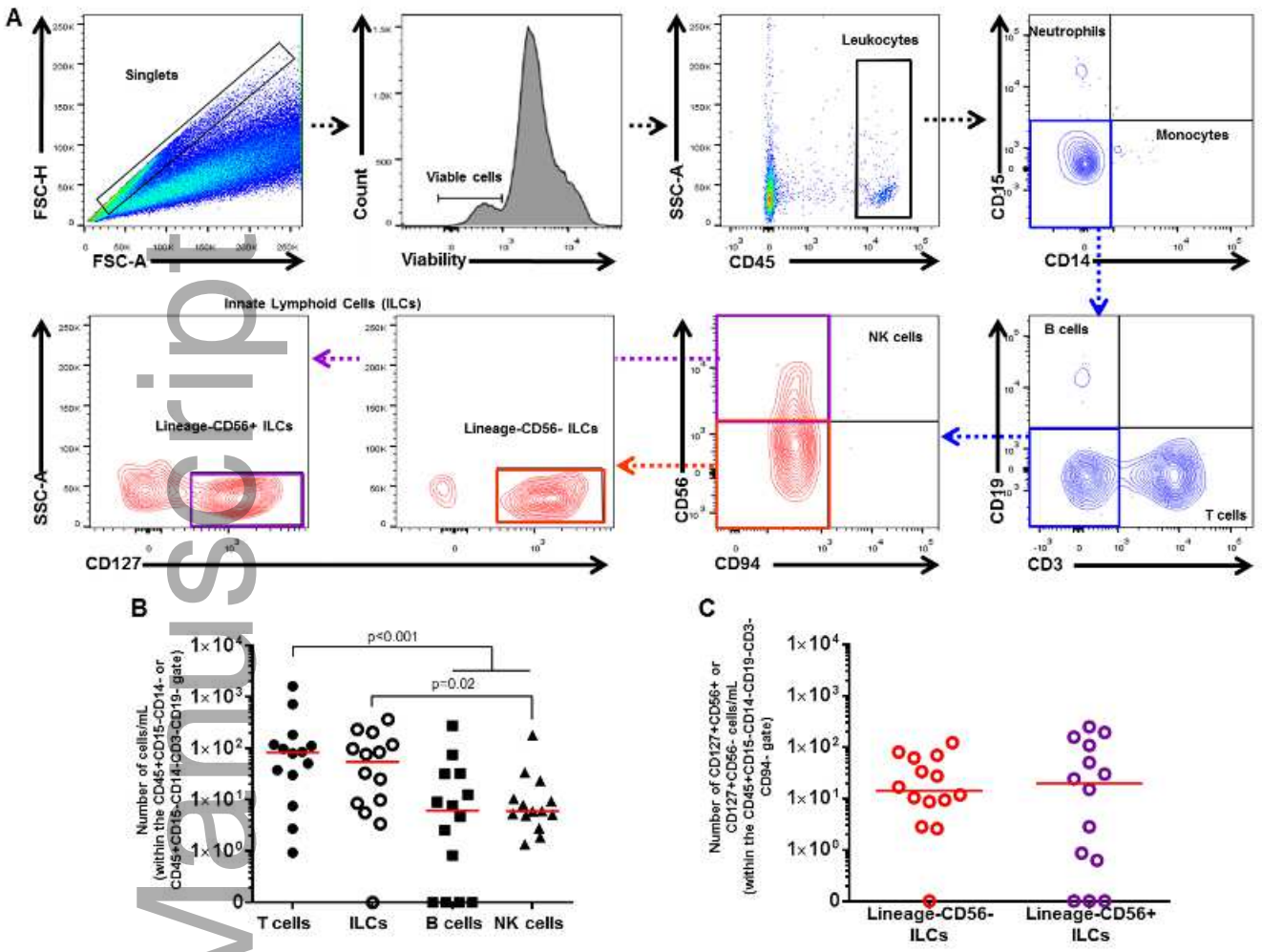

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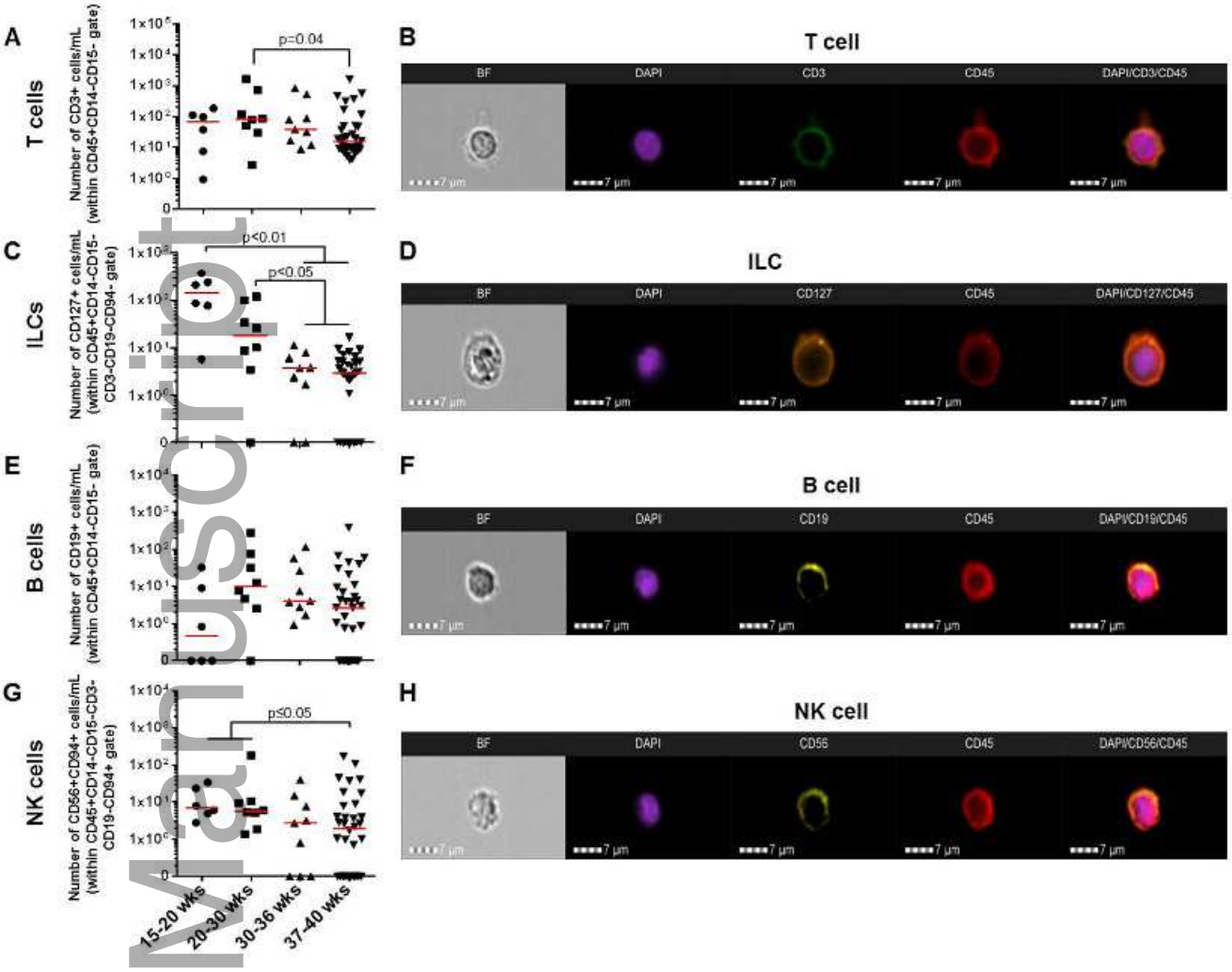

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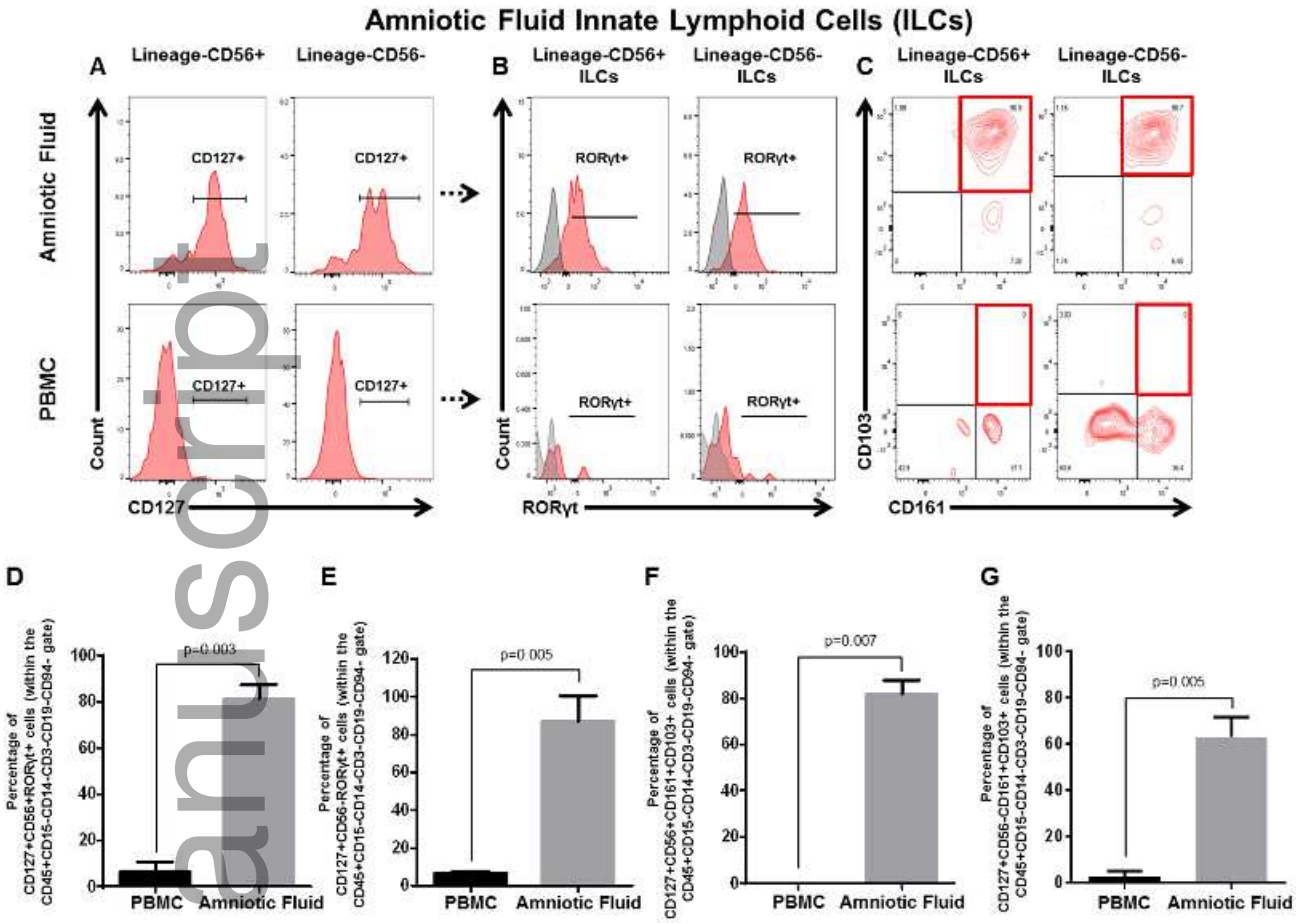

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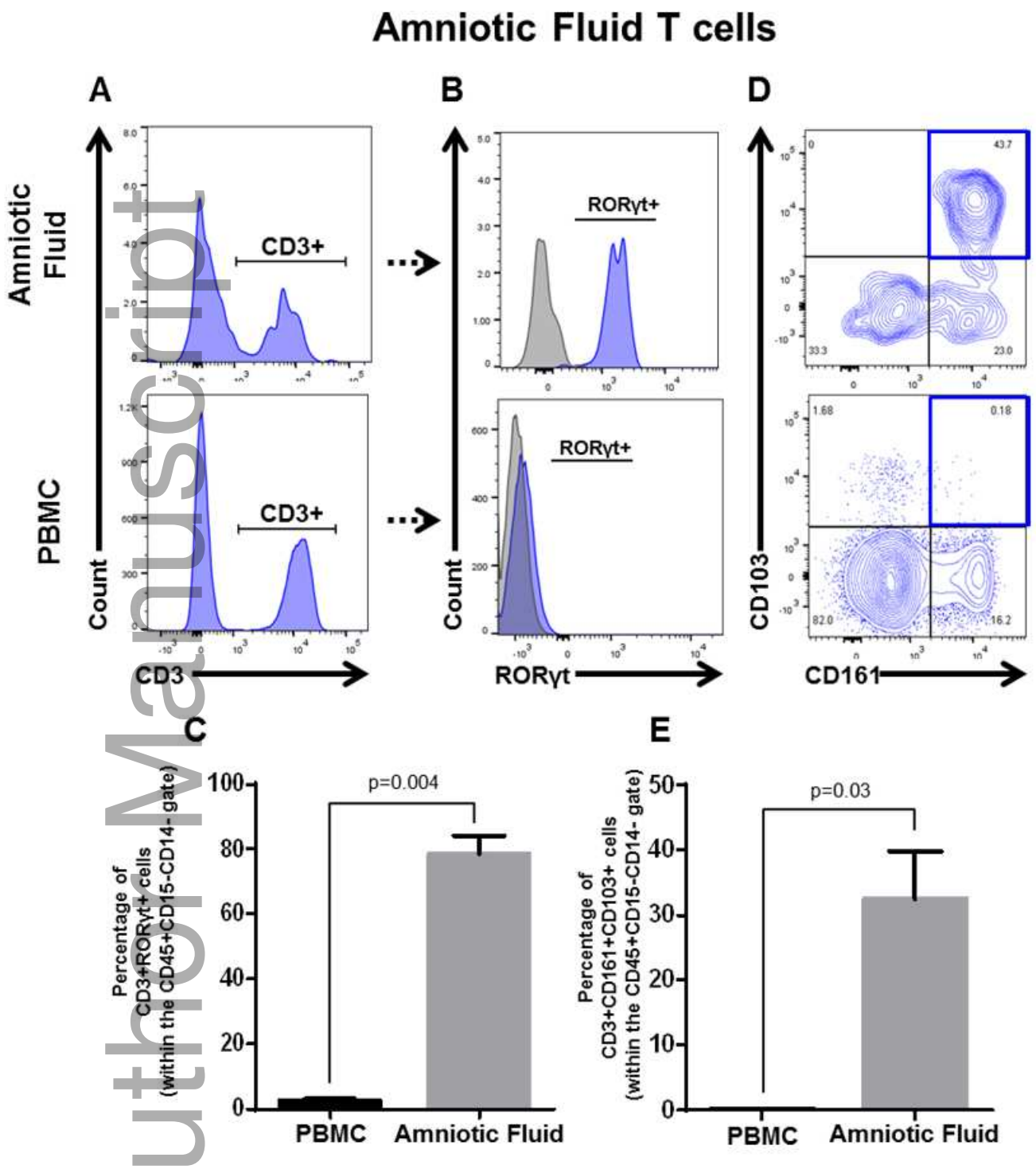

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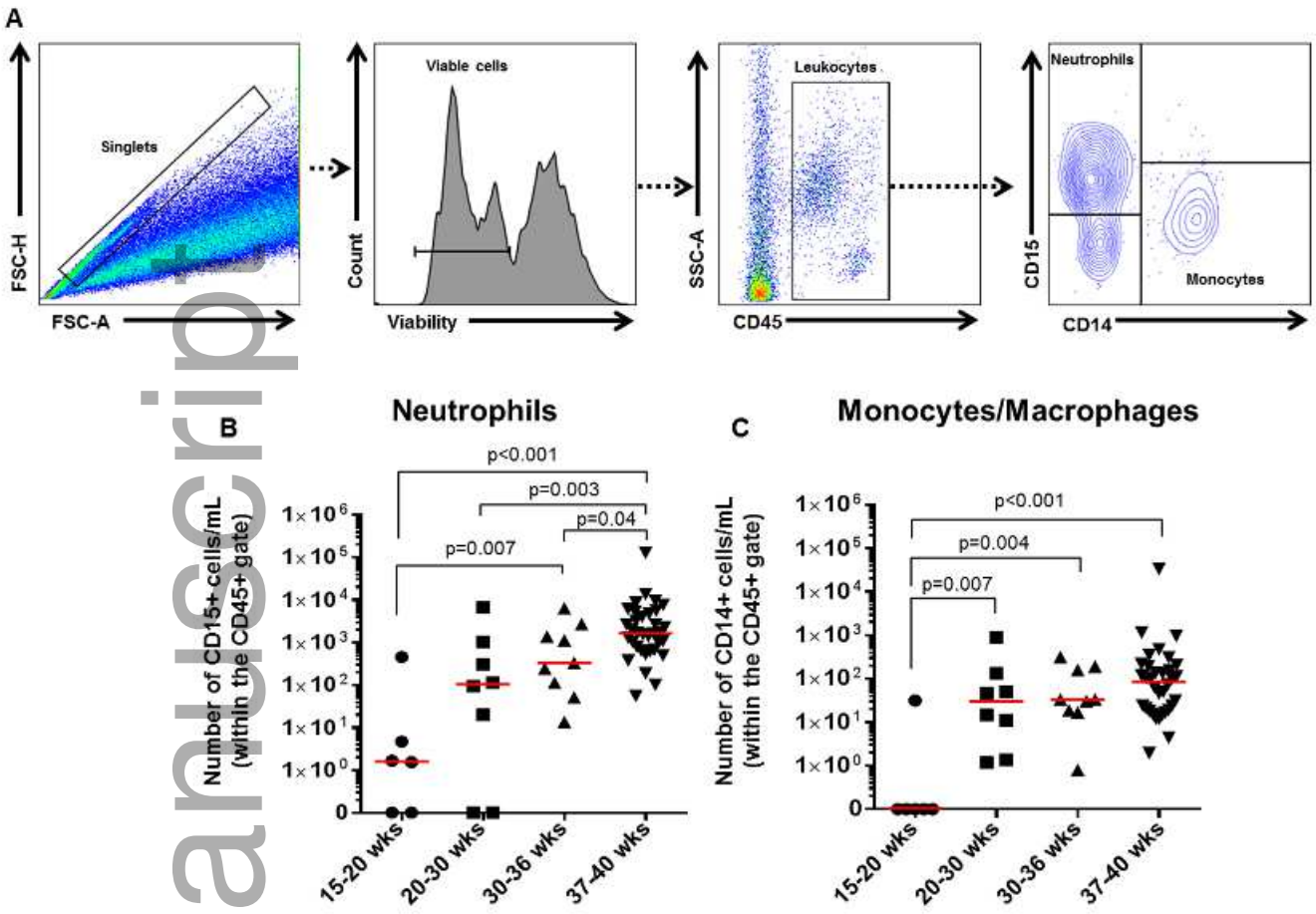

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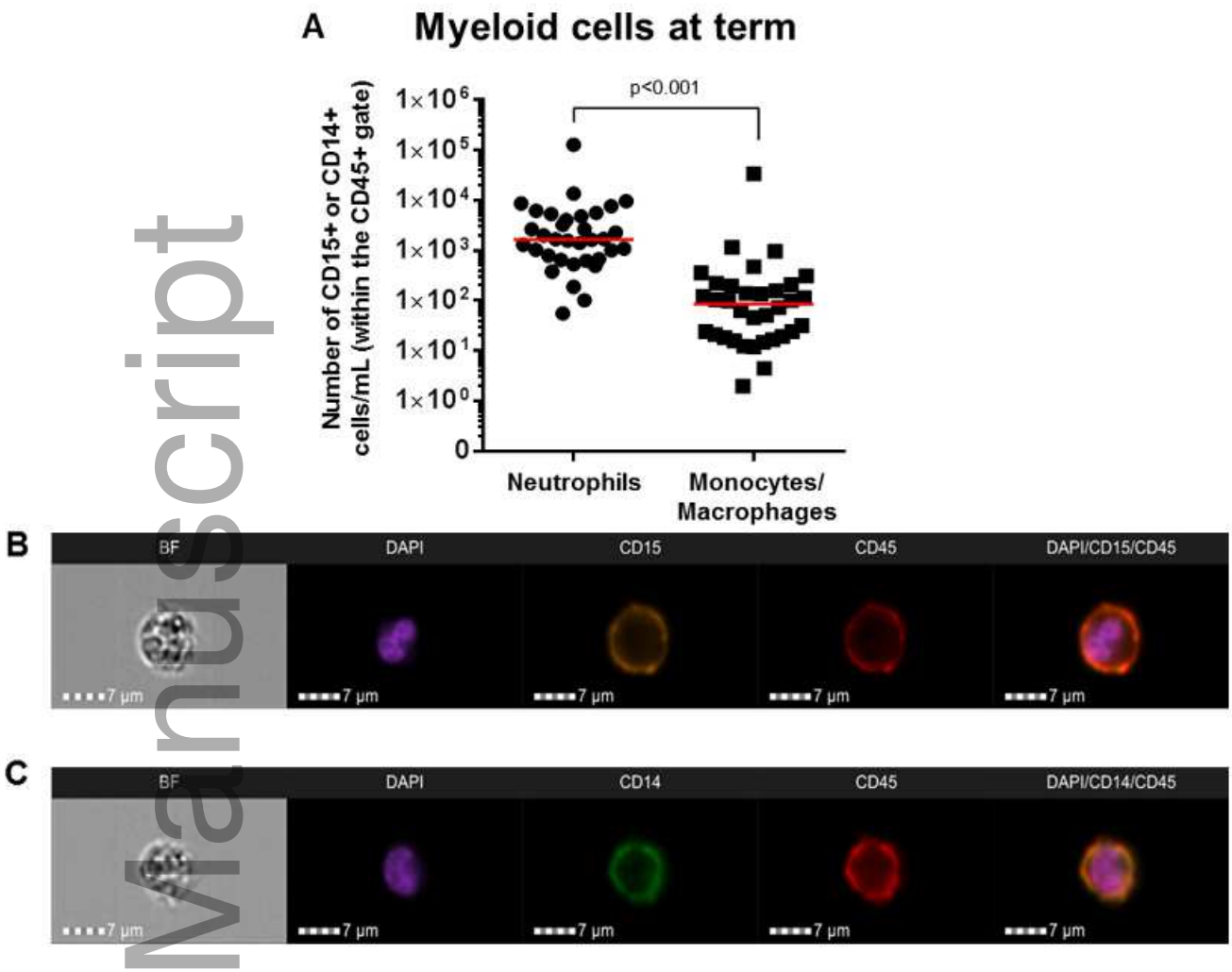

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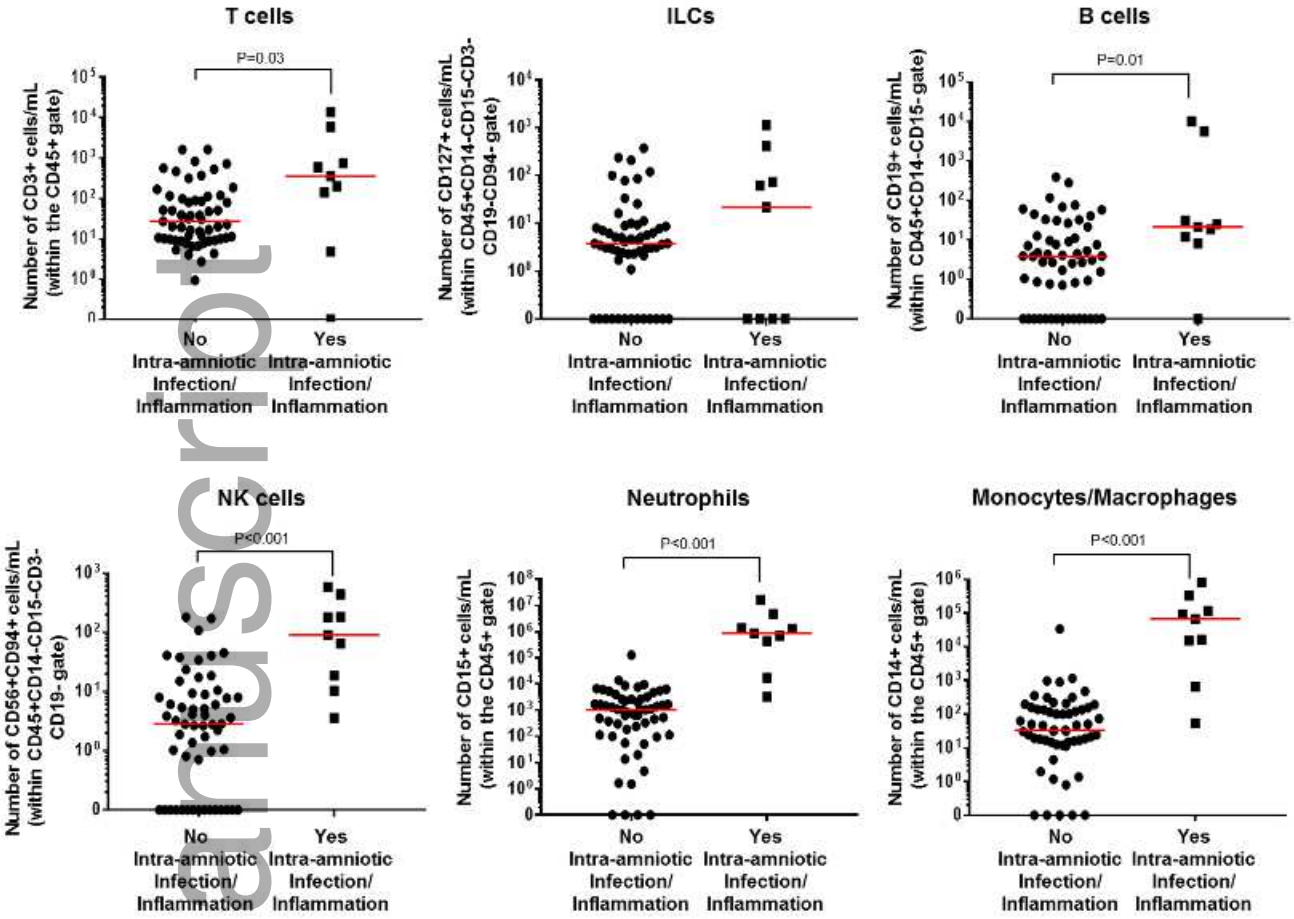

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