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Are B Cells Altered in the Decidua of Women with Preterm or Term Labor?

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

ABSTRACT

Problem: The immunophenotype of B cells at the maternal-fetal interface (decidua) in labor at term and preterm labor is poorly understood.

Method of study: Decidual tissues were obtained from women with preterm or term labor and from non-labor gestational age-matched controls. Immunophenotyping of decidual B cells was performed using multi-color flow cytometry.

Results: 1) In the absence of acute or chronic chorioamnionitis, total B cells were more abundant in the decidua parietalis of women who delivered preterm than those who

delivered at term, regardless of the presence of labor; 2) decidual transitional and naïve B cells were the most abundant B-cell subsets; 3) decidual B1 B cells were increased in women with labor at term or preterm labor and chronic chorioamnionitis compared to those without this placental lesion; 4) decidual transitional B cells were reduced in women with preterm labor compared to those without labor; 5) naïve, class-switched, and non-class-switched B cells in the decidual tissues underwent mild alterations with the process of preterm labor and/or placental inflammation; 6) decidual plasmablasts seemed to increase in women with labor at term or preterm labor with chronic chorioamnionitis; and 7) decidual B cells expressed high levels of interleukin (IL)-12, IL-6 and/or IL-35.

Conclusions: Total B cells are not increased with the presence of preterm or term labor; yet, specific subsets (B1 and plasmablasts) undergo alterations in women with chronic chorioamnionitis. Therefore, B cells are solely implicated in the pathological process of preterm labor in a subset of women with chronic inflammation of the placenta. These findings provide insight into the immunology of the maternal-fetal interface in preterm and term labor.

Keywords: B1 B cells, chronic chorioamnionitis, funisitis, memory B cells, naïve B cells, placental inflammation, plasmablasts, pregnancy, transitional B cells

INTRODUCTION

Preterm labor, which commonly precedes preterm birth [1, 2], is a syndrome involving multiple pathological processes [3, 4]. Among the known mechanisms, pathological inflammation is the best-characterized causal link to preterm labor and birth [5-14]. To date, the most studied causes of pathological inflammation leading to preterm labor have been 1) intra-amniotic infection/inflammation resulting from microbial invasion of the amniotic cavity [5, 7, 8, 15-33], and 2) intra-amniotic inflammation without detectable microorganisms (i.e. sterile intra-amniotic inflammation) identified by using both molecular and conventional microbiological techniques [32, 34-38], proposed to be due to endogenous danger signals, or alarmins [39-49]. Most research concerning inflammation-induced preterm labor has therefore focused on the innate limb of immunity [50-73]. Yet, several studies reported strong evidence that T cells, the primary

cellular component of the adaptive immune system, are present at the maternal-fetal interface [74-89]. More recently, we provided evidence indicating that T cells are also implicated in the mechanisms that lead to labor at term [84, 85] and spontaneous preterm labor [90-97]. However, B cells, the main humoral component of adaptive immunity, have been less investigated.

B cells were first described in the placental bed of women early in gestation [98], which was confirmed by later studies [99, 100]. During early pregnancy, B cells are implicated in the mechanisms of maternal-fetal tolerance [101-115]. Decidual B cells modestly increased between 27 and 33 weeks of gestation followed by a slight decline at term [100]. In the absence of labor at term, multiple studies reported that B cells are present at the human maternal-fetal interface (i.e. decidua basalis and decidua parietalis) [76, 77, 99, 116, 117]. Moreover, B cells seem to be increased in the decidua basalis [77], but not in the decidua parietalis [77, 116], during the physiological process of labor at term. A recent study provided evidence indicating a role for B cells in the pathogenesis of preterm labor: the results showed increased proportions of B cells in the decidua parietalis of women who underwent spontaneous preterm labor compared to those with labor at term [118]. However, this study did not include gestational age-matched controls, allowing for further investigation of the B-cell compartment at the human maternal-fetal interface in both labor at term and preterm labor.

In the current study, we performed immunophenotyping of the decidua basalis and decidua parietalis of women who underwent the physiological process of labor at term or the syndrome of preterm labor leading to preterm birth. Decidual tissues from gestational age-matched controls were also included. In addition, the B-cell subsets in acute and chronic maternal inflammatory lesions of the placenta were compared. Lastly, the production of cytokines by decidual B cells was determined.

MATERIALS AND METHODS

Human subjects, clinical specimens, and definitions

Samples of the human placental basal plate (maternal side of the placenta, decidua basalis) and chorioamniotic membranes (amnion, chorion, and decidua parietalis) were collected from patients within 30 minutes after delivery at Hutzel Women's Hospital in the Detroit Medical Center, Detroit, MI, USA, in partnership with

Wayne State University School of Medicine 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), Detroit, MI, USA. The collection and utilization of biological materials for research purposes were approved by the Institutional Review Boards of Wayne State University and NICHD. All participating women provided written informed consent prior to the collection of samples.

The study groups included women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). Preterm birth was defined as delivery before 37 weeks of gestation. Labor was defined by the presence of regular uterine contractions at a frequency of at least 2 contractions every 10 minutes with cervical changes resulting in delivery. The TIL and PTL study groups were subdivided based on the presence of acute histologic chorioamnionitis (ACA) and chronic histologic chorioamnionitis (CCA) (see "Placental histopathological examination" section for diagnostic criteria). Patients with neonates having congenital or chromosomal abnormalities were excluded from this study. The clinical and demographic characteristics of the study population are shown in Tables 1 and 2. Both the decidua basalis and decidua parietalis were collected from most patients; however, the decidua basalis was not available in a few cases. Therefore, Table 1 describes patients from which the decidua basalis was available, and Table 2 describes patients from which the decidua parietalis was available for experiments.

Placental histopathological examination

Placentas were examined histologically by a perinatal pathologist blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols. 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 [94, 119-122]. Maternal acute placental inflammation was defined by the

infiltration of neutrophils into the chorion and amnion, termed acute histologic chorioamnionitis (ACA) [119, 121]. Maternal chronic histologic chorioamnionitis (CCA) was defined as lymphocytic infiltration into the chorionic trophoblast layer or chorioamniotic connective tissue [90, 94].

Decidual leukocyte isolation

Decidual leukocytes were isolated from the decidual tissue of patients from each study group as previously described [123]. Briefly, the decidua basalis was collected from the basal plate of the placenta, and the decidua parietalis was separated from the chorioamniotic membranes. The decidual tissues were homogenized in StemPro Accutase Cell Dissociation Reagent (Life Technologies, Grand Island, NY, USA) using a gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA). Homogenized tissues were incubated in Accutase for 45 min at 37℃ with gentle agitation. After incubation, tissues were washed in 1X phosphate-buffered saline (PBS; Life Technologies) and filtered through a 100-μm cell strainer (Fisher Scientific, Durham, NC, USA). The resulting cell suspensions were centrifuged at 300 x g for 10 min at 4℃. The decidual mononuclear cells were then separated using a density gradient (Ficoll-Paque Plus; GE Healthcare Biosciences, Piscataway, NJ, USA) in accordance with the manufacturer's instructions. The cells collected from the mononuclear layer of the density gradient were washed with 1X PBS and immediately used for immunophenotyping.

Immunophenotyping of decidual B cells

Mononuclear cell suspensions from decidual tissues were stained with the LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific/Molecular Probes, Eugene, OR, USA) prior to immunophenotyping. Mononuclear cell suspensions were then washed with stain buffer (CAT#554656; BD Biosciences, San Jose, CA, USA) and incubated with 20 μL of human FcR Blocking Reagent (CAT#130-059-901; Miltenyi Biotec) in 80 μL of stain buffer for 10 min at 4℃. The cells were incubated with extracellular fluorochrome-conjugated anti-human monoclonal antibodies for 30 min at 4℃ in the dark (Supplementary Table 1). Stained cells were washed and resuspended in 0.5 mL of FACS staining buffer and acquired using an LSRII flow cytometer and

FACSDiva 6.0 software (BD Biosciences). The absolute number of cells was determined using CountBright absolute counting beads (Life Technologies, Molecular Probes). The analysis and figures were performed using FlowJo software version 10 (FlowJo, LLC, Ashland, OR, USA). Lymphocytes were gated using forward scatter (FSC) verse side scatter (SSC). B cells were gated as $CD19^{+}CD3^{-}$ cells within the lymphocytic and viability gates (Figure 1B). The cell surface markers (Supplementary Table 1) used to identify the different B-cell subsets were determined by an extensive literature search (Table 3). Cytokine expression by decidual B cells was also performed using specific monoclonal antibodies directly after isolation from the tissue without stimulation (Supplementary Table 1).

Statistics

Statistical analyses were conducted using SPSS software version 19.0 (IBM Corporation, Armonk, NY, USA). The Mann-Whitney *U*-test was used for comparisons between the two study groups. Two-tailed (p-values without an asterisk) or one-tailed (p-values with an asterisk) p-values were reported. The Friedman test was used for comparisons between decidual tissues and blood samples collected from the same patients. For patient demographics, the Kruskall-Wallis test was performed for continuous variables and the Fisher's exact test for nominal variables. A p-value <0.05 was considered statistically significant.

RESULTS

In the absence of acute or chronic chorioamnionitis, total B cells are more abundant in the decidua parietalis of women who delivered preterm than in those who delivered at term, regardless of the process of labor

Figure 1A shows the spatial localization of the decidua basalis and decidua parietalis. The gating strategy used to identify total B cells is shown in Figure 1B. First, we compared the proportion of total B cells in the decidual tissues to those in the maternal blood and cord blood. The frequency of B cells in the decidua basalis and decidua parietalis was lower than that observed in maternal blood and cord blood (Figure 1C). Most of the B cells present in the decidua basalis and decidua parietalis co-

expressed CD20, as did the B cells in maternal blood and cord blood (Figure 1D). Additionally, a $CD19^{+}CD20^{-}$ subset was present in decidual tissues but rarely found in maternal blood and cord blood (Figure 1D). The number of B cells in the decidua basalis and decidua parietalis ranged from $2x10^2$ to $2x10^5$ cells (Supplementary Figure 1), considerably lower than previously reported [118]. Yet, our finding is consistent with previous studies indicating that the B-cell population is a small fraction among decidual leukocytes [85, 123]. Preterm placentas are significantly smaller than term placentas and the amount of decidual tissue available varies among patients; therefore, flow cytometry quantification may not allow calculation of the absolute number of decidual B cells. For this reason, we used frequencies, also referred to as proportions, throughout the study.

In the decidua basalis, the total number of B cells did not vary among the term and preterm groups (TNL vs TIL vs PTNL vs PTL, Figure 1E). Further, no differences were observed among those groups when samples were allocated into subgroups comparing acute and chronic inflammatory lesions of the placenta (Figure 1F). In the decidua parietalis, the total number of B cells did not vary among term and preterm groups when placental inflammation was not considered (TNL vs TIL vs PTNL vs PTL, Figure 1G). In the absence of acute or chronic chorioamnionitis, the decidua parietalis of women who delivered preterm had higher proportions of B cells than those who delivered at term, regardless of the presence of labor (PTNL or PTL vs TNL or TIL, Figure 1H). The data indicate that B cells are more abundant in preterm gestation than at term, consistent with findings previously reported [100]. Yet, contrary to what has been reported [77, 118], the processes of preterm and term labor did not alter the frequency of total B cells. In the decidua parietalis, B cells were more abundant in women with chronic chorioamnionitis who underwent labor at term compared to those without this placental lesion (TIL with CCA vs TIL or TIL with ACA, Figure 1H). However, no differences were observed in B-cell frequencies between the PTL and PTL with CCA groups.

Identification of different B-cell subsets in the decidual tissues

Next, we performed an extensive literature review of the markers used to identify the main B-cell subsets (Table 3). Two main B-cell subsets have been described: B1

and B2 B cells [124] (Figure 2A). Both B1 and B2 B cells can become transitional B cells, naïve B cells, class-switched memory B cells, non-class-switched memory B cells, and plasmablasts [124] (Figure 2B). $CD19^{+}$ B cells were subdivided into the above-mentioned B-cell subsets. The gating strategy used to identify these B-cell subsets is shown in Figure 2C. We found that B2 B cells were more abundant in the decidual tissues than B1 B cells, which made up a distinct but very small population (data not shown). Therefore, the B1 B cells were considered by themselves. A t-SNE plot showing the different B-cell subsets identified in the decidual tissues is shown in Figure 2D. Transitional and naïve B cells were the most abundant subsets in the decidual tissues (Figure 2D).

B1 B cells are increased in the decidual tissues of women with chronic chorioamnionitis who underwent labor at term or preterm labor

B1 B cells were rarely found in the decidual tissues, as shown in the t-SNE plot (Figure 3A). In the decidua basalis, the frequency of B1 B cells did not change between the labor and no labor groups (TNL vs TIL and PTNL vs PTL, Figure 3B). A higher proportion of B1 B cells was observed in women who underwent preterm labor compared to those who underwent labor at term (PTL vs TIL, Figure 3B). This difference was most likely driven by the presence of chronic chorioamnionitis in the preterm labor group (PTL with CCA, Figure 3C). The presence of chronic chorioamnionitis also increased the proportion of B1 B cells in labor at term (TIL with CCA vs TIL or TIL with ACA, Figure 3C). In the decidua parietalis, B1 B cells were less abundant in women who underwent labor at term compared to those who delivered at term without labor (TIL vs TNL, Figure 3D). However, women who underwent labor at term with chronic chorioamnionitis had higher proportions of B1 B cells than those without this placental lesion (TIL with CCA vs TIL, Figure 3E). Women who underwent preterm labor with chronic chorioamnionitis also had higher proportions of B1 B cells compared to those without this placental lesion (PTL with CCA vs PTL or PTL with ACA, Figure 3B). These results show that B1 B cells are increased in the decidual tissues of women who underwent labor at term or preterm labor in the presence of chronic

chorioamnionitis, but not in those with acute chorioamnionitis or without placental lesions.

Transitional B cells were reduced in the decidual tissues of women with preterm labor compared to gestational age-matched controls

Transitional B cells are the critical link between bone marrow immature and mature B cells present in the peripheral repertoire [125, 126]. The transitional B-cell subset is thought to represent a key negative selection checkpoint for autoreactive B cells [127, 128]. Transitional B cells were one of the most abundant B-cell subsets present in the decidual tissues, as shown in the t-SNE plot (Figure 4A). In the decidua basalis, the proportion of transitional B cells was lower in women who underwent preterm labor compared to those who delivered preterm in the absence of labor (PTL vs PTNL, Figure 4B). Additionally, transitional B cells were less abundant in women who underwent preterm labor with or without acute or chronic chorioamnionitis compared to those who delivered preterm without labor (PTL with CCA, PTL with ACA, and PTL vs PTNL, Figure 4C). However, transitional B cells were more abundant in women who delivered preterm without labor than in those who delivered at term in the absence of labor (PTNL vs TNL, Figure 4B). In the decidua parietalis, transitional B cells were less abundant in women who underwent preterm labor than in those who delivered preterm without labor (PTL vs PTNL, Figure 4D). Transitional B cells were even fewer in women who underwent preterm labor with chronic chorioamnionitis than in those without this placental lesion (PTL with CCA vs PTL, Figure 4E). Taken together, these data show that transitional B cells are reduced in women with preterm labor compared to gestational age-matched controls.

Naïve B cells undergo mild alterations in the decidual tissues

Naïve B cells were another abundant B-cell subset found in the decidual tissues, as shown in the t-SNE plot (Figure 5A). In the decidua basalis, naïve B cells did not vary among the study groups (TNL vs TIL vs PTNL vs PTL, Figure 5B). In the absence of placental lesions, naïve B cells were modestly higher in women who delivered preterm without labor than in those who delivered at term without labor (PTNL vs TNL, Figure

5C). In the decidua parietalis, naïve B cells were reduced in women who underwent preterm labor compared to those who delivered preterm without labor (PTL vs PTNL, Figure 5D). This reduction was driven by the presence of chronic chorioamnionitis given that women who underwent preterm labor with this placental lesion tended to have fewer naïve B cells in the decidua parietalis (Figure 5E). Women with preterm labor and chronic chorioamnionitis had fewer naïve B cells than those with labor at term and this placental lesion (PTL with CCA vs TIL with CCA, Figure 5E). In summary, naïve B cells undergo mild alterations in the decidua basalis of women with preterm labor and chronic chorioamnionitis.

Class-switched and non-class-switched memory B cells are rare and undergo mild alterations in the decidual tissues

Naïve B cells can undergo class-switch recombination generating class-switched or non-class-switched memory B cells [129]. Next, we examined whether these B-cell subsets were present in the decidual tissues. Class-switched and non-class-switched memory B cells were rarely present in the decidual tissues (Figures 6A and 7A). In the decidua basalis, class-switched memory B cells were more abundant in women who underwent preterm labor than in those with labor at term (PTL vs TIL, Figure 6B). This increase was most likely due to the presence of chronic chorioamnionitis since women who underwent preterm labor with this placental lesion tended to have higher proportions of class-switched memory B cells than those without it (PTL with CCA vs PTNL, PTL, or PTL with ACA, Figure 6C). In the absence of placental lesions, class-switched memory B cells were less abundant in women who underwent labor at term (TIL vs TNL, Figure 6C). However, class-switched memory B cells were more abundant in women who underwent labor at term with chronic chorioamnionitis than in those with acute chorioamnionitis (TIL with CCA vs TIL with ACA, Figure 6C). In the decidua parietalis, class-switched memory B cells were less abundant in women with labor at term than in those who delivered at term without labor (TIL vs TNL, Figure 6D), which was consistently observed in the absence of placental lesions (Figure 6E). No differences were observed between women who delivered preterm with and without labor (PTNL vs PTL, Figure 6D). In summary, in the absence of placental lesions, class-

switched memory B cells are rare in the decidual tissues and undergo mild alterations with the presence of labor and/or placental inflammation.

Non-class-switched memory B cells were even less abundant than class-switched memory B cells in the decidual tissues (Figure 7A). In the decidua basalis, non-class-switched memory B cells did not vary among the study groups (TNL vs TIL vs PTNL vs PTL, Figure 7B). When the patients were divided into subgroups according to the presence of placental lesions, a slight increase in the proportion of non-class-switched memory B cells was observed in women who underwent labor at term with chronic chorioamnionitis compared to those with acute chorioamnionitis (TIL with CCA vs TIL with ACA, Figure 7C). In the decidua parietalis, non-class-switched memory B cells were less abundant in women who underwent preterm labor than those who delivered preterm without labor (PTL vs PTNL, Figure 7D). This was most likely driven by the presence of chronic chorioamnionitis since women with this placental lesion had lower proportions of this B-cell subset than those without it (PTL with CCA vs PTL and PTNL, Figure 7E). In the absence of placental lesions, a small reduction in the proportion of non-class-switched memory B cells was also observed in women with labor at term compared to those without labor (TIL vs TNL, Figure 7E). However, women who underwent labor at term with chronic chorioamnionitis had greater proportions of non-class-switched memory B cells than those without this placental lesion (TIL with CCA vs TIL, Figure 7E). Therefore, in the absence of placental lesions, non-class-switched memory B cells are rare in the decidual tissues and undergo mild alterations with the presence of labor and/or placental inflammation.

Plasmablasts seemed to increase in the decidual tissues of women with chronic chorioamnionitis who underwent labor at term or preterm labor

Naïve B cells and memory B cells can differentiate into plasmablasts [129, 130]. We then investigated the presence of plasmablasts in the decidual tissues. Plasmablasts were a relatively abundant population in the decidual tissues (Figure 8A). In the decidua basalis and decidua parietalis, plasmablasts did not vary among the study groups (TNL vs TIL vs PTNL vs PTL, Figures 8B & 8D). When the samples were divided into subgroups according to the presence of placental lesions, we consistently found that

women who underwent labor at term with chronic chorioamnionitis had higher proportions of plasmablasts than those without this placental lesion in both the decidua basalis and decidua parietalis (TIL with CCA vs TIL or TIL with ACA, Figures 8C & 8E). Plasmasblasts seemed to be more abundant in women who underwent preterm labor with CCA than in those without this placental lesion (Figures 8C & 8E). Taken together, these data show that plasmablasts are abundant in the decidual tissues of women who underwent labor at term or preterm labor with chronic chorioamnionitis.

B cells in the decidual tissues expressed high levels of IL-12, IL-6 and/or IL35

Recent studies indicated that, besides production of antibodies, B cells participate in immune responses by producing inflammatory cytokines [131, 132]. We investigated whether decidual B cells produce pro- and anti-inflammatory cytokines that are associated with the process of labor. In the decidua basalis and decidua parietalis, a small proportion of B cells expressed cytokines (Figure 9A & 9C). In the decidua basalis, B cells expressed higher levels of interleukin (IL)-12 and IL-35 than IFNγ, IL-2, IL-4, IL-6, IL-10, and TNFα (Figure 9B). In the decidua parietalis, B cells expressed higher levels of IL-12 and IL-6 than other cytokines (Figure 9D). These results show that B cells participate in the inflammatory milieu at the maternal-fetal interface.

DISCUSSION

Principal findings

The principal findings of this study are as follows: 1) In the absence of acute or chronic chorioamnionitis, total B cells were more abundant in the decidua parietalis of women who delivered preterm than in those who delivered at term, regardless of the presence of labor; 2) decidual transitional and naïve B cells were the most abundant B-cell subsets; 3) decidual B1 B cells were increased in women with labor at term or preterm labor with chronic chorioamnionitis compared to those without this placental lesion; 4) decidual transitional B cells were reduced in women with preterm labor compared to those without labor; 5) decidual naïve B cells underwent mild alterations with the process of preterm labor and/or placental inflammation; 6) class-switched and non-class-switched memory B cells were rare and underwent mild alterations with the

process of labor and/or placental inflammation; 7) decidual plasmablasts seemed to increase in women with labor at term or preterm with chronic chorioamnionitis; and 8) decidual B cells expressed high levels of IL-12, IL-6, or IL-35**.** These findings indicate that specific B-cell populations at the maternal-fetal interface undergo alterations in a subset of women with labor at term and preterm labor and chronic chorioamnionitis.

In the absence of acute or chronic chorioamnionitis, total B cells were more abundant in the decidua parietalis of women who delivered preterm than in those who delivered at term, regardless of the presence of labor

Herein, we found that total B cells were not increased in the decidua basalis and decidua parietalis of women who underwent labor at term compared to those who delivered at term without labor. This finding is consistent, in part, with what has previously been published. We and other investigators have reported that there are no differences in the proportion of B cells in the decidua parietalis of women who delivered at term with and without labor [77, 85]. However, previous studies have shown that the decidua basalis of women who underwent spontaneous labor at term had greater proportions of total B cells than those who delivered at term without labor [77]. The discrepancy between the above-mentioned study and our findings could be explained by the fact that we considered only viable B cells. These findings show that the frequency of total viable B cells is not altered with the physiological process of labor at term.

We also found that total B cells were not increased in the decidua basalis and decidua parietalis of women who underwent preterm labor compared to those who delivered preterm in the absence of labor. To our knowledge, it is the first time that this comparison between preterm labor cases and gestational age-matched controls has been made. A previous study reported that the abundance of B cells in the decidua parietalis was significantly increased in women who underwent spontaneous preterm labor compared to those who underwent spontaneous labor at term [118]. However, this comparison warrants caution given that gestational age may influence the proportion of B cells found in the decidual tissues [100]. Without considering acute and chronic chorioamnionitis, no differences in the frequency of total B cells in the decidua parietalis

were observed between women who underwent preterm labor and those with term labor. Women with preterm labor, but without acute or chronic chorioamnionitis, had higher frequencies of decidual B cells compared to those with labor at term. Nonetheless, this increase in the proportion of total B cells was also observed by comparing women who delivered preterm in the absence of labor to those who delivered at term without labor. Therefore, these data indicate that the process of preterm labor does not increase the proportion of total B cells in the decidua parietalis; rather, it is an effect of gestational age.

After the two study groups were subdivided based on the presence of acute or chronic chorioamnionitis, we found that the frequency of total B cells in the decidua basalis did not significantly vary. Yet, differences in B-cell proportions in the decidua parietalis were observed among the study groups. This result is explained by the fact that acute and chronic chorioamnionitis are diagnosed in the chorioamniotic membranes, located next to the decidua parietalis [94, 121]. Of interest, the presence of chronic chorioamnionitis increased the frequency of total B cells in the decidua parietalis of women with labor at term but not in those with preterm labor (Figure 1H). These findings suggest that the process of chronic chorioamnionitis in labor at term is distinct from that observed in women with preterm labor.

Different B-cell subsets were present in the decidual tissues; yet, we focused our discussion on the main differences observed in women with labor at term and those with preterm labor.

Decidual B1 B cells are increased in women with chronic chorioamnionitis who underwent labor at term or preterm labor

B1 B cells show a skewed antigen receptor repertoire toward self-antigens, as well as tonic B-cell receptor intracellular signaling, spontaneous secretion of IgM, and efficient T-cell stimulation [133]. Herein, we found that B1 B cells are increased in the decidua basalis and decidua parietalis of women with labor at term or preterm labor with chronic chorioamnionitis. These data suggest, for the first time, a role for decidual B1 B cells in the pathological process of chronic chorioamnionitis associated with either labor at term or preterm labor. Potentially, B1 B cells could be participating in the stimulation

of $CD8^+$ cytotoxic T cells, which can induce apoptosis of trophoblasts leading to maternal anti-fetal rejection [94]. More recently, we found that both decidual $CD4^+$ and $CD8^+$ T cells from women who underwent spontaneous preterm labor displayed an effector memory phenotype and expressed high levels of granzyme B and perforin [97], molecules capable of cytotoxicity [134-140]. B1 B cells constitutively secrete IL-10 [141], suggesting that these cells are implicated in the regulation of such a chronic inflammatory process associated with labor at term and preterm labor. Given that B1 B cells are either pro-inflammatory (through cytotoxic $CD8^+$ T-cell stimulation) or anti-inflammatory (through IL-10 secretion), their role may vary in chronic chorioamnionitis and requires further investigation.

Decidual naïve B cells undergo mild alterations with the process of preterm labor and/or placental inflammation

During a primary response, transitional B cells differentiate to naïve B cells that upon a secondary response can generate memory B cells [142]. Naïve B cells and memory B cells display differential expression of IgD or CD27 and *in vitro* function following BCR stimulation [143-145]. Overall, naïve B cells present antigen-inexperienced responses compared to memory B cells [145]. Naïve B cells can also proliferate into short-lived plasmablasts or plasma cells that produce low-affinity antibodies [146]. In the current study, we found that naïve B cells were reduced in patients with preterm labor and chronic chorioamnionitis, but no differences were observed in memory B cells. Yet, decidual tissues from women who underwent preterm labor with chronic chorioamnionitis tended to have greater proportions of plasmablasts. These data suggest that, in women with preterm labor and chronic chorioamnionitis, naïve B cells might proliferate to plasmablasts in the decidual tissues. However, additional investigation of decidual B cells is required to strengthen this proposed concept.

Decidual plasmablasts increase in women who underwent labor at term or preterm labor with chronic chorioamnionitis

Plasmablasts are newly differentiated B cells that can leave the lymphoid organs and home in either tissue or bone marrow [147, 148]. Additionally, they are capable of

differentiating into fully mature plasma cells [149]. Plasmablasts are usually short-lived and can be generated inside and outside of the germinal centers [150]. In general, plasmablasts represent an accessible source of mature antibodies characterized by the regulation of several transcription factors, such as BLIMP1 (B-lymphocyte-induced maturation protein 1) and IRF4 (interferon-regulatory factor 4) [151]. Our results showed that plasmablasts seemed to increase in women who underwent labor at term or preterm labor with chronic chorioamnionitis. These data suggest that, in addition to T cells, the hallmark of chronic chorioamnionitis [94], B-cell antibody-mediated responses are implicated in the chronic inflammatory process associated with preterm labor and birth.

B cells can express cytokines in the decidual tissues

Cytokine production by B cells is implicated in multiple aspects of immunity [132]. Thus, B cells can express pro- and anti-inflammatory cytokines to mediate immune responses [131, 132]. Herein, we found that, in both the decidua basalis and decidua parietalis, B cells expressed high levels of IL-12, a cytokine that increased in the amniotic fluid of women with preterm labor and preterm birth [46]. In the decidua parietalis, B cells also expressed high levels of IL-6, a cytokine released by gestational tissues and highly relevant in the pathological process of preterm labor [26, 152]. Therefore, these data provide evidence that, by releasing cytokines, B cells at the maternal-fetal interface contribute to the pro-inflammatory microenvironment associated with labor. B cells, however, could also be participating in the regulation of such a hostile microenvironment, given that these cells also expressed a high level of IL-35, an immunosuppressive cytokine previously reported to be expressed by the placental tissues [153]. Further studies are needed to investigate whether decidual B cells display different cytokine profiles than those derived from gestational-aged matched controls.

Conclusion

The current study provides the characterization of B-cell subsets at the human maternal-fetal interface during labor at term and preterm labor. In the absence of acute or chronic chorioamnionitis, total B cells are more abundant in the decidua parietalis of

women who delivered preterm than in those who delivered at term, regardless of the process of labor. Yet, an increase in the proportions of B1 B cells and plasmablasts was observed in women who underwent labor at term or preterm labor with chronic chorioamnionitis compared to those without this placental lesion. Decidual B cells are capable of producing pro- and anti-inflammatory cytokines. In conclusion, the B-cell compartment at the maternal-fetal interface undergoes alterations in women with labor at term or preterm labor and chronic chorioamnionitis, suggesting that these adaptive immune cells are implicated in the process of labor associated with chronic inflammation of the placenta. Additional experimentation is required to investigate the functionality of chronic chorioamnionitis-derived B cells.

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FIGURE LEGENDS

Figure 1. Immunophenotyping of B cells in the decidua basalis and decidua parietalis. (A) Representation of the spatial localization of the decidua basalis and decidua parietalis. **(B)** Flow cytometry gating strategy used to identify B cells in the decidual tissues. Lymphocytes were gated using forward scatter (FSC) versus side scatter (SSC). B cells were gated as $CD19^{+}CD3^{-}$ cells within the viability and lymphocytic gates. **(C)** The proportion of $CD19^{+}$ B cells in samples of case-matched decidua basalis, decidua parietalis, maternal blood, and cord blood (n = 5). **(D)** Co-expression of CD20 by $CD19^{+}$ B cells in the decidua basalis, decidua parietalis, maternal blood, and cord blood. The proportions of $CD19^{+}$ B cells in the decidua basalis **(E)** or decidua parietalis **(G)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of $CD19^{+}$ B cells in the decidua basalis **(F)** or decidua parietalis **(H)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 2. B-cell subsets in the decidua basalis and decidua parietalis. (A) Schematic representation of B1 and B2 B cells. **(B)** Schematic representation of the B-cell subsets identified in the decidual tissues. **(C)** Flow cytometry gating strategy used to identify the following B-cell subsets: B1 B cells ($CD3^{-}CD19^{+}CD20^{+}CD27^{+}CD43^{+}$ cells); transitional B cells ($CD3^{-}CD19^{+}CD20^{+}CD38^{hi}CD24^{hi}$ cells); naïve B cells ($CD3^{-}CD19^{+}CD20^{+}CD27^{-}IgD^{+}$ cells); class-switched memory B cells ($CD3^{-}CD19^{+}CD20^{+}CD27^{+}IgD^{-}$ cells); non-class-switched memory B cells ($CD3^{-}CD19^{+}CD20^{+}CD27^{+}IgD^{+}$ cells); and plasmablasts ($CD3^{-}CD19^{+}CD20^{-}CD38^{+}CD24^{-}$ cells). **(D)** A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing B-cell subsets in the decidual tissues. Red = B1 B cells, purple = transitional B cells, orange = naïve B cells, blue = class-switched memory B cells, green = non-

class-switched memory B cells, turquoise = plasmablasts, and grey = other $CD19^+$ B cells.

Figure 3. B1 B cells in the decidua basalis and decidua parietalis. (A) A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing B1 B cells in the decidual tissues. Red = B1 B cells and grey = other $CD19^+$ B cells. The proportions of B1 B cells in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of B1 B cells in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 4. Transitional B cells in the decidua basalis and decidua parietalis. (A) A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing transitional B cells in the decidual tissues. Red = transitional B cells and grey = other $CD19^+$ B cells. The proportions of transitional B cells in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of transitional B cells in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 5. Naïve B cells in the decidua basalis and decidua parietalis. (A) A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing

naïve B cells in the decidual tissues. Red = naïve B cells and grey = other $CD19^+$ B cells. The proportions of naïve B cells in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of naïve B cells in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 6. Class-switched memory B cells in the decidua basalis and decidua parietalis. (A) A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing class-switched memory B cells in the decidual tissues. Red = class-switched memory B cells and grey = other $CD19^+$ B cells. The proportions of class-switched memory B cells in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of class-switched memory B cells in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 7. Non-class-switched memory B cells in the decidua basalis and decidua parietalis. (A) A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing non-class-switched memory B cells in the decidual tissues. Red = non-class-switched memory B cells and grey = other $CD19^+$ B cells. The proportions of non-class-switched memory B cells in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who

delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of non-class-switched memory B cells in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 8. Plasmablasts in the decidua basalis and decidua parietalis. **(A)** A representative t-distributed stochastic neighbor embedding (t-SNE) dot plot visualizing plasmablasts in the decidual tissues. Red = plasmablasts, blue = $CD19^{+}CD20^{-}$ B cells, and grey = other $CD19^{+}$ B cells. The proportions of plasmablasts in the decidua basalis **(B)** or decidua parietalis **(D)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. The TIL and PTL patients were subdivided into those with acute histologic chorioamnionitis (ACA) or chronic histologic chorioamnionitis (CCA), and those without these lesions. Non-labor controls without ACA or CCA were included as well. The proportions of plasmablasts in the decidua basalis **(C)** or decidua parietalis **(E)** in these patient subgroups. N = 4 – 16 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Figure 9. Cytokine expression by decidual B cells. (A & C) Staggered offset overlay histograms of cytokines expressed by B cells ($CD45^{+}CD3^{-}CD19^{+}CD20^{+}$ cells) in the decidua basalis and parietalis. Four representative samples of decidual B cells isolated from women who delivered preterm or term. Control histograms represent signals derived from isotypes or autofluorescence controls. **(B & D)** Proportions of B cells expressing IFNγ, IL-2, IL-4, IL-6, IL-10, IL-12, IL-35, and TNFα in the decidua basalis and parietalis.

Supplementary Figure 1. B-cell counts in the decidua basalis and decidua parietalis. The number of $CD19^{+}$ B cells in the decidua basalis **(A)** and decidua

parietalis **(B)** from women who delivered at term with labor (TIL) or without labor (TNL) and women who delivered preterm with labor (PTL) or without labor (PTNL). N = 6 – 37 per group. Red midlines and whiskers indicate medians and interquartile ranges, respectively.

Table 1. Clinical and demographic characteristics of the patient population used to perform immunophenotyping of the decidua basalis

	Term without labor (n=15)	Term labor (n=14)	Term labor with ACA (n=10)	Term labor with CCA (n=13)	Preterm without labor (n=4)	Preterm labor (n=8)	Preterm labor with ACA (n=4)	Preterm labor with CCA (n=14)	p-value
Maternal age (years; median [IQR])[a]	24 (21-29)	25 (21.3-30.3)	23.5 (22-25.8)	24 (22-29)	30.5 (28-31)	26 (20.5-29)	34 (29.5-37)	24.5 (21-29.8)	0.5
Body mass index (kg/m^2; median [IQR])[a]	31.8 (29.5-34.8)	30.2 (25.3-36.8)	29 (26.6-33.2)	31.3 (26.6-37.3)	34.2 (31.8-37.1)	30.2 (21.7-41.1)[c]	28.3 (25.2-34.7)	24.3 (21.6-38.2)	0.7
Primiparity[b]	13.3% (2/15)	28.6% (4/14)	40% (4/10)	23.1% (3/13)	0% (0/4)	12.5% (1/8)	0% (0/4)	14.3% (2/14)	0.6
Race[b]									0.6
African-American	73.3% (11/15)	78.6% (11/14)	90% (9/10)	92.3% (12/13)	75% (3/4)	62.5% (5/8)	75% (3/4)	78.6% (11/14)	
Caucasian	13.3% (2/15)	21.4% (3/14)	0% (0/10)	7.7% (1/13)	25% (1/4)	25% (2/8)	25% (1/4)	14.3% (2/14)	
Asian	0% (0/15)	0% (0/14)	10% (1/10)	0% (0/13)	0% (0/4)	0% (0/8)	0% (0/4)	0% (0/14)	
Other	13.3% (2/15)	0% (0/14)	0% (0/10)	0% (0/13)	0% (0/4)	12.5% (1/8)	0% (0/4)	7.1% (1/14)	
Cesarean section	100% (15/15)	14.3% (2/14)	20% (2/10)	7.7% (1/13)	100% (4/4)	37.5% (3/8)	25% (1/4)	21.4% (3/14)	<0.001
Gestational age at delivery (weeks; median [IQR])[a]	39.1 (39-39.4)	39 (38.2-40.2)	39.4 (38.4-39.8)	39.6 (38.9-40.1)	34.7 (33.1-36.1)	34.4 (31-35.5)	34 (32.8-34.7)	34.9 (33.3-36.3)	<0.001

Birthweight (grams)[a]	3225 (2930-3482.5)	3515 (3121.3-3645)	3392.5 (3348.8-3628.8)	3460 (3155-3665)	2025.5 (1392-2798.8)	1932.5 (1401.5-3233.8)	2130 (1487.5-2776.3)	2240 (1887.5-2322.5)	<0.001

Data are given as median (interquartile range, IQR) and percentage (n/N). ACA = acute chorioamnionitis; CCA = chronic chorioamnionitis (please see Methods for definitions).

[a]Kruskal-Wallis test.

[b]Fisher's exact test.

[c]One missing data.

Table 2. Clinical and demographic characteristics of the patient population used to perform immunophenotyping of the decidua parietalis

	Term without labor (n=16)	Term labor (n=14)	Term labor with ACA (n=10)	Term labor with CCA (n=14)	Preterm without labor (n=4)	Preterm labor (n=9)	Preterm labor with ACA (n=4)	Preterm labor with CCA (n=14)	p-value
Maternal age (years; median [IQR])[a]	24 (21-28.8)	25 (21.3-30.3)	23.5 (22-25.8)	24 (22-29)	30.5 (28-31)	28 (21-32)	34 (29.5-37)	24.5 (21-29.8)	0.5
Body mass index (kg/m^2; median [IQR])[a]	31.5 (29.5-34.4)[c]	30.2 (25.3-36.8)	29 (26.6-33.2)	32.8 (26.7-37.1)	34.2 (31.8-37.1)	32.9 (21.9-40)[c]	28.3 (25.2-34.7)	24.3 (21.6-38.2)	0.7
Primiparity[b]	12.5% (2/16)	28.6% (4/14)	40% (4/10)	21.4% (3/14)	0% (0/4)	11.1% (1/9)	0% (0/4)	14.3% (2/14)	0.6
Race[b]									0.5
African-American	75% (12/16)	78.6% (11/14)	90% (9/10)	92.9% (13/14)	75% (3/4)	55.6% (5/9)	75% (3/4)	78.6% (11/14)	
Caucasian	18.8% (3/16)	21.4% (3/14)	0% (0/10)	7.1% (1/14)	25% (1/4)	33.3% (3/9)	25% (1/4)	14.3% (2/14)	
Asian	0% (0/16)	0% (0/14)	10% (1/10)	0% (0/14)	0% (0/4)	0% (0/9)	0% (0/4)	0% (0/14)	
Other	6.2% (1/16)	0% (0/14)	0% (0/10)	0% (0/14)	0% (0/4)	11.1% (1/9)	0% (0/4)	7.1% (1/14)	
Cesarean section	100% (16/16)	14.3% (2/14)	20% (2/10)	7.1% (1/14)	100% (4/4)	33.3% (3/9)	25% (1/4)	21.4% (3/14)	<0.001

Gestational age at delivery (weeks; median [IQR])[a]	39.1 (39-39.2)	39 (38.2-40.2)	39.4 (38.4-39.8)	39.7 (38.9-40.1)	34.7 (33.1-36.1)	34.7 (31.3-35.9)	34 (32.8-34.7)	34.9 (33.3-36.3)	<0.001
Birthweight (grams)[a]	3222.5 (2940-3456.3)	3515 (3121.3-3645)	3392.5 (3348.8-3628.8)	3510 (3206.3-3706.3)	2025.5 (1392-2798.8)	2160 (1435-3155)	2130 (1487.5-2776.3)	2240 (1887.5-2322.5)	<0.001

Data are given as median (interquartile range, IQR) and percentage (n/N). ACA = acute chorioamnionitis; CCA = chronic chorioamnionitis (please see Methods for definitions).

[a]Kruskal-Wallis test.

[b]Fisher's exact test.

[c]One missing data.

Table 3. Markers to identify B cells subsets

B cell subsets	Immunophenotype	References
B1 B cells	$CD19^+CD27^+CD43^+$	Griffin DO et.al[1, 2], Torring C et.al[3], Inui M et.al[4]
B2 B cells	$CD19^+CD23^+$ $CD27^-CD43^-$	Griffin DO et.al[1], Deng C et.al[5]
Transitional B cells	$CD19^+CD38^{hi}CD24^{hi}$	Marie-Cardine A et.al[6], Ha Y et.al[7], Seifert M et.al[8], de Masson A et.al[9], Cherukuri A et.al[10], Heidt S et.al[11], Latorre I et.al[12], Tebbe B et.al[13], Luk F et.al[14], Demoersman J et.al[15], Li S et.al[16]
Naïve B cells	$CD19^+CD27^-IgD^+$	Guerreiro-Cacais A et.al[17], So N et.al[18], Heath E et.al[19], Cantaert T et.al[20], Toapanta F et.al[21], Jansen M et.al[22], Castaneda D et.al[23], Wu X et.al[24], Nakayama Y et.al[25]
Class-switched memory B cells	$CD19^+CD27^+IgD^-$	Anolik J et.al[26], Tian C et.al[27], Ghannam A et.al[28], Palanichamy A et.al[29], Berkowska M et.al[30], Morbach H et.al[31], Wu Y et.al[32], So N et.al[18], Heath E et.al[19], Topanta F et.al[21], Labuda L et.al[33], Degauque N et.al[34], Zhang L et.al[35], Bagnara D et.al[36], Czarnowicki T et.al[37], Hayashi M et.al[38], Mensah F et.al[39], Woda M et.al[40], Castaneda D et.al[23], Martins C et.al[41]
Non class-switched memory B cells	$CD19^+CD27^+IgD^+$	Anolik J et.al[26], Tian C et.al[27], Palanichamy A et.al[29], Colonna-Romano G et.al[42], Jacobi A et.al[43], Wu Y et.al[32], So N et.al[18], Heath E et.al[19], Topanta F et.al[21], Weller S et.al[44], Labuda LA et.al[33], Clemente A et.al[45], Czarnowicki T et.al[37], Mensah F et.al[39], Castaneda D et.al[23], Martins C et.al[41], Corneth O et.al[46], Torigoe M et.al[47], Hu F, et.al[48]
Plasmablasts	$CD19^+CD20^-$ $CD38^+CD24^-$	Morbach H et.al[49], Lin W et.al[50], Benett M et.al[51]

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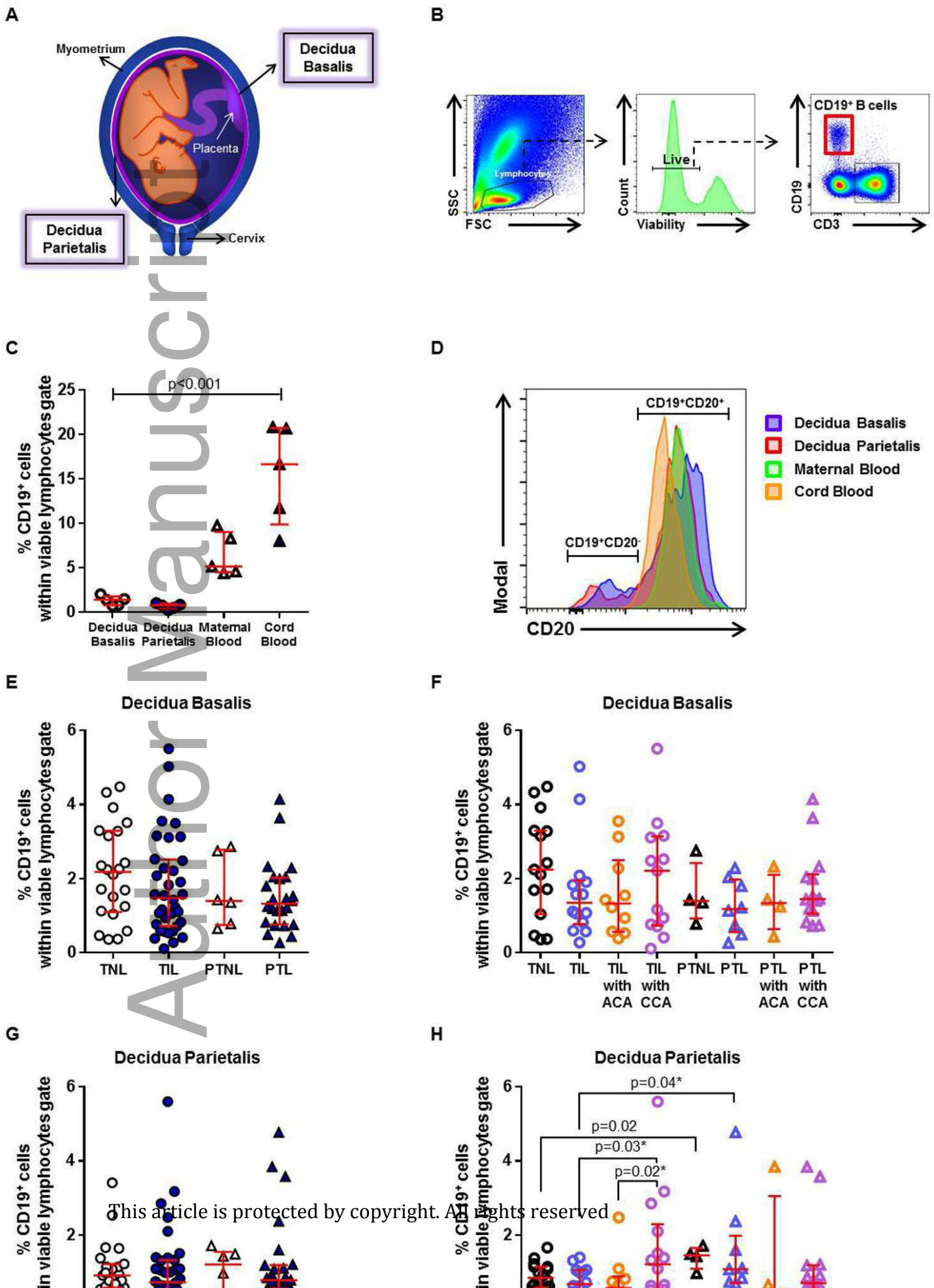

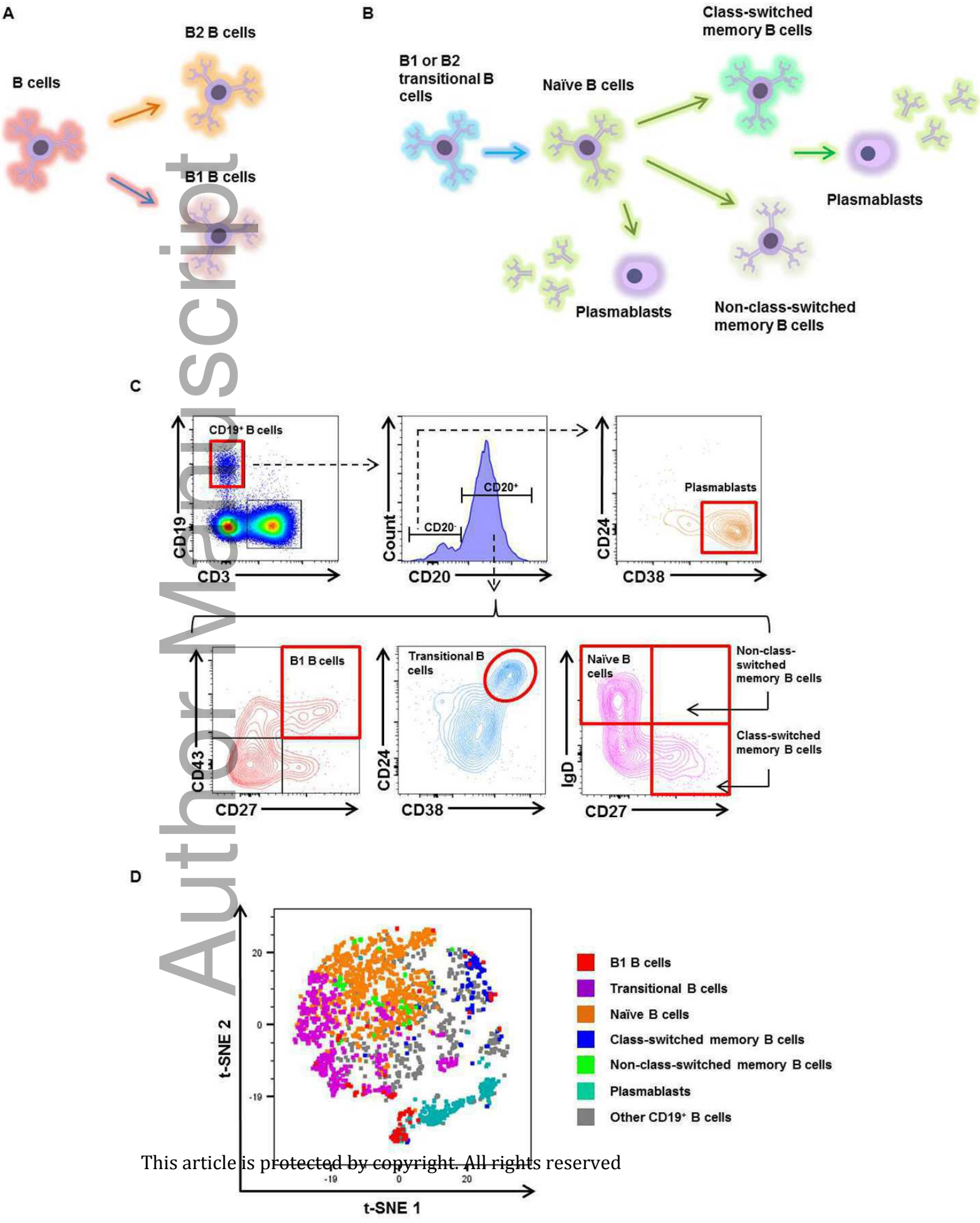

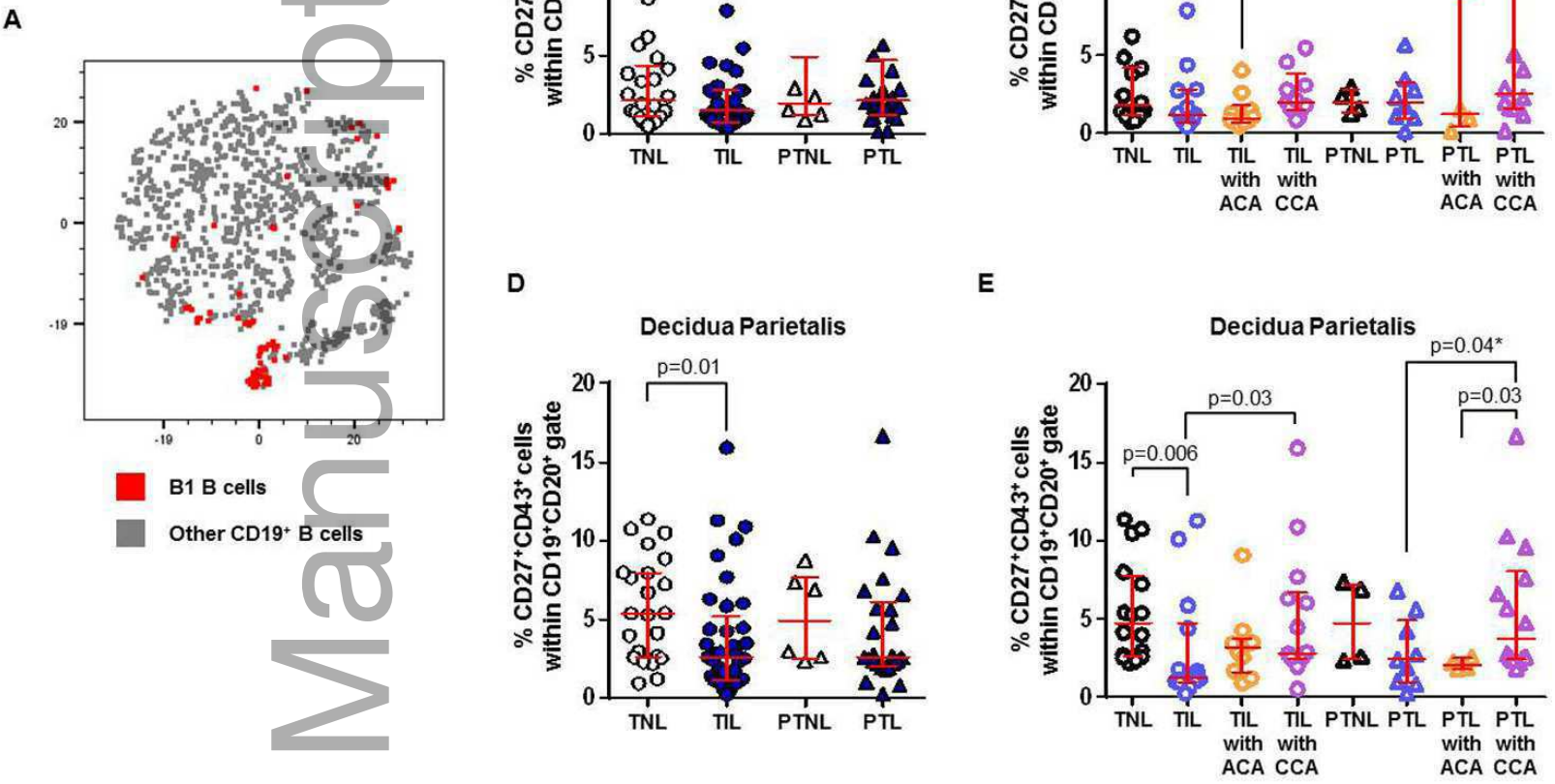

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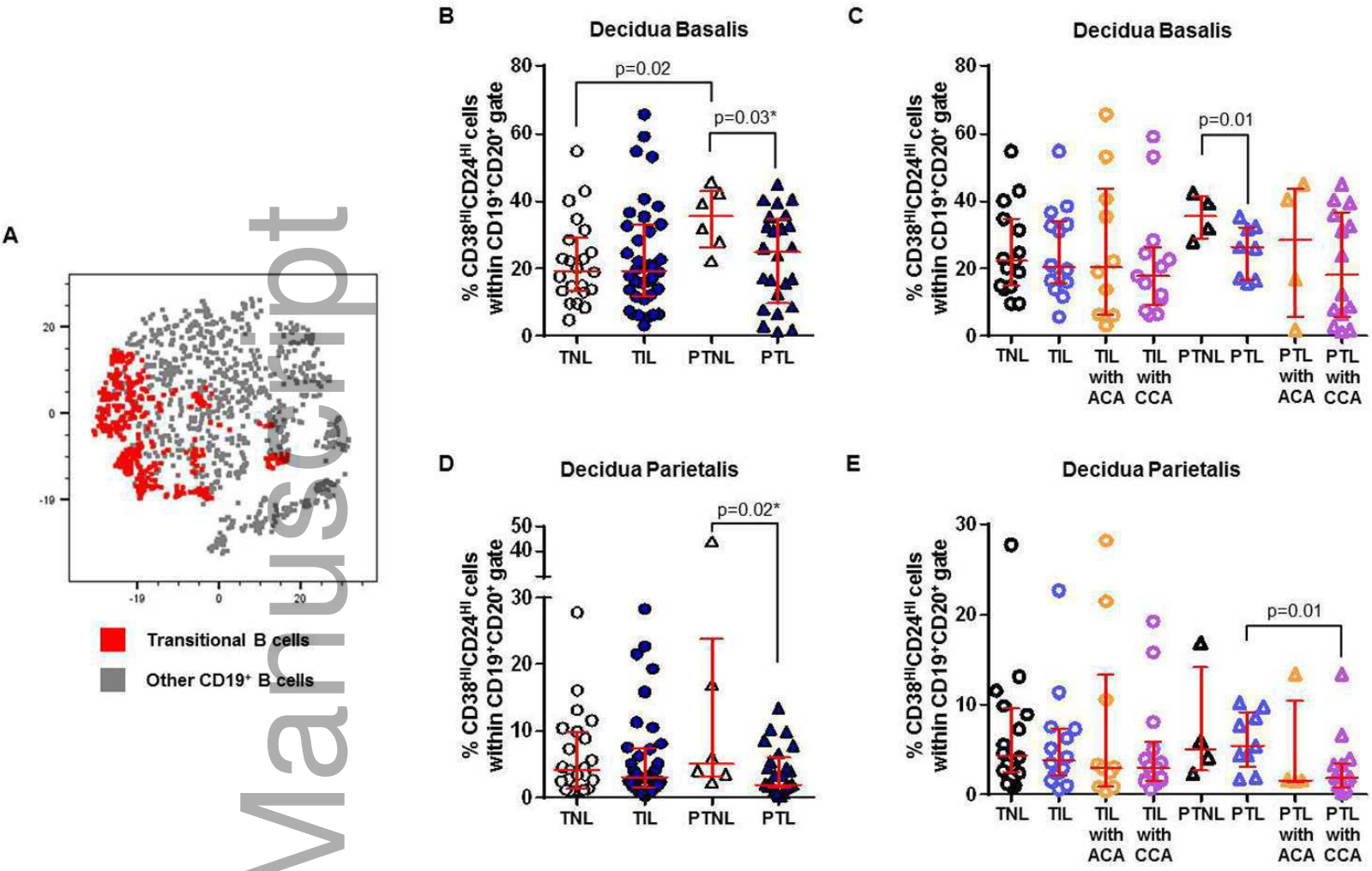

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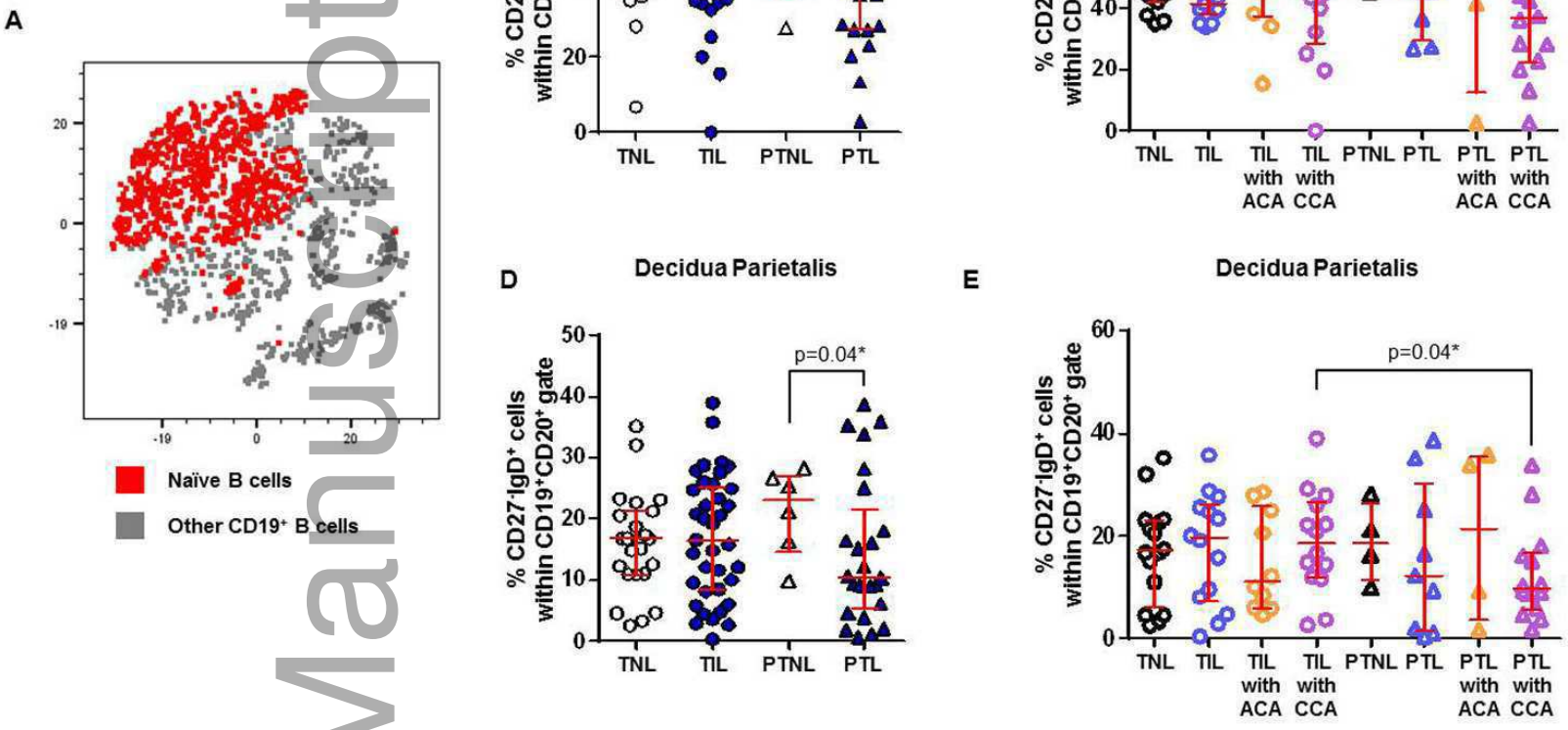

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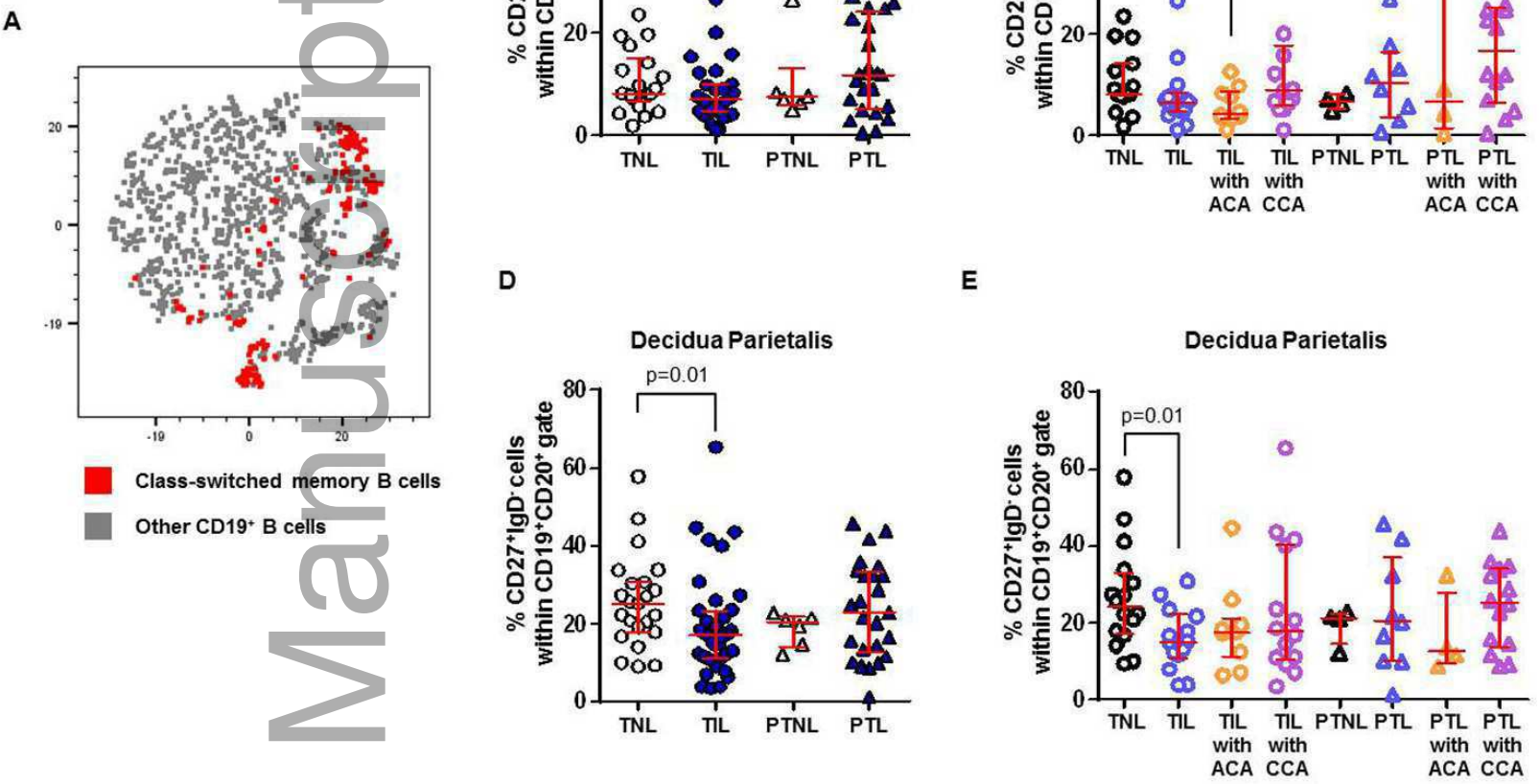

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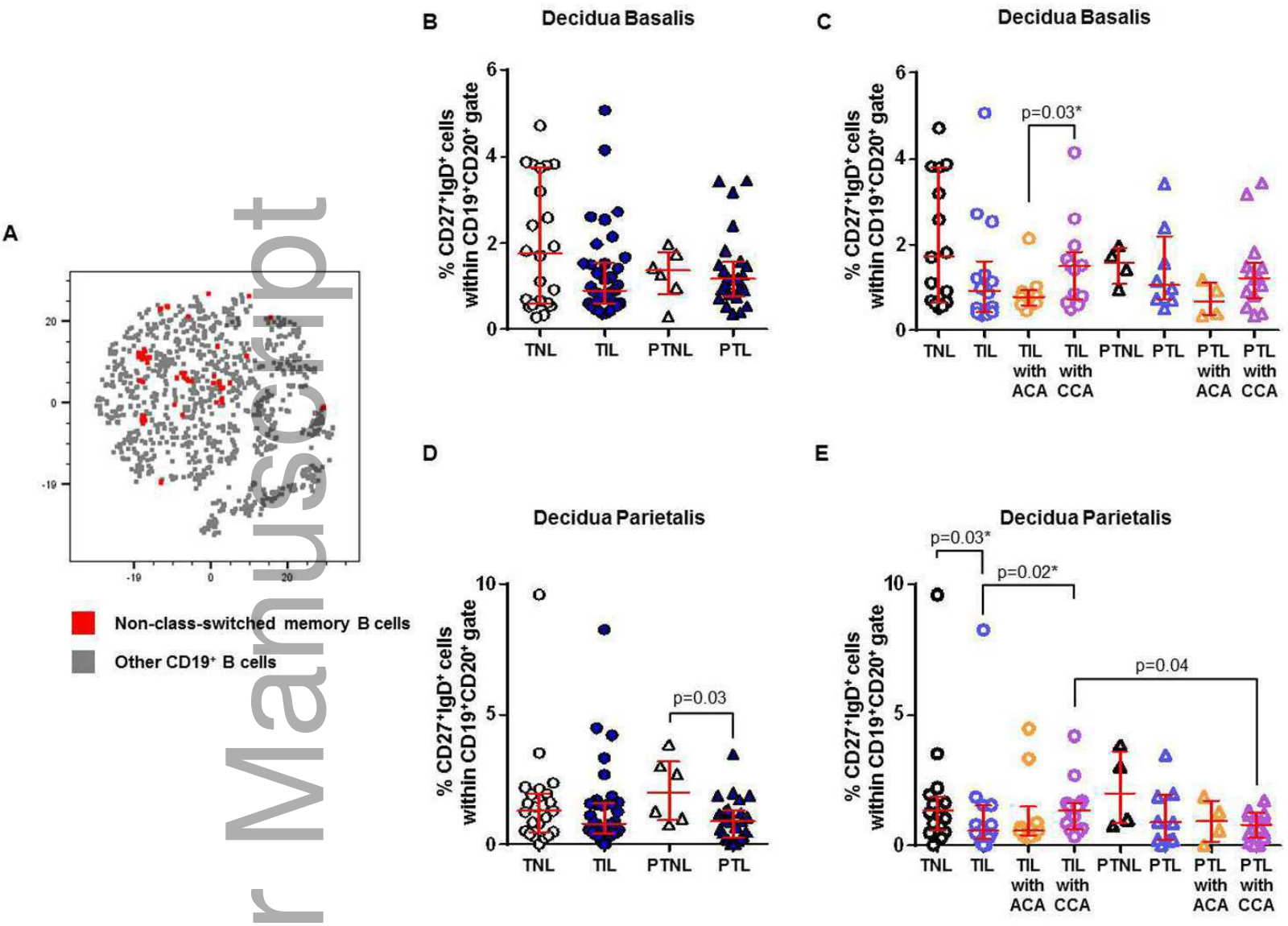

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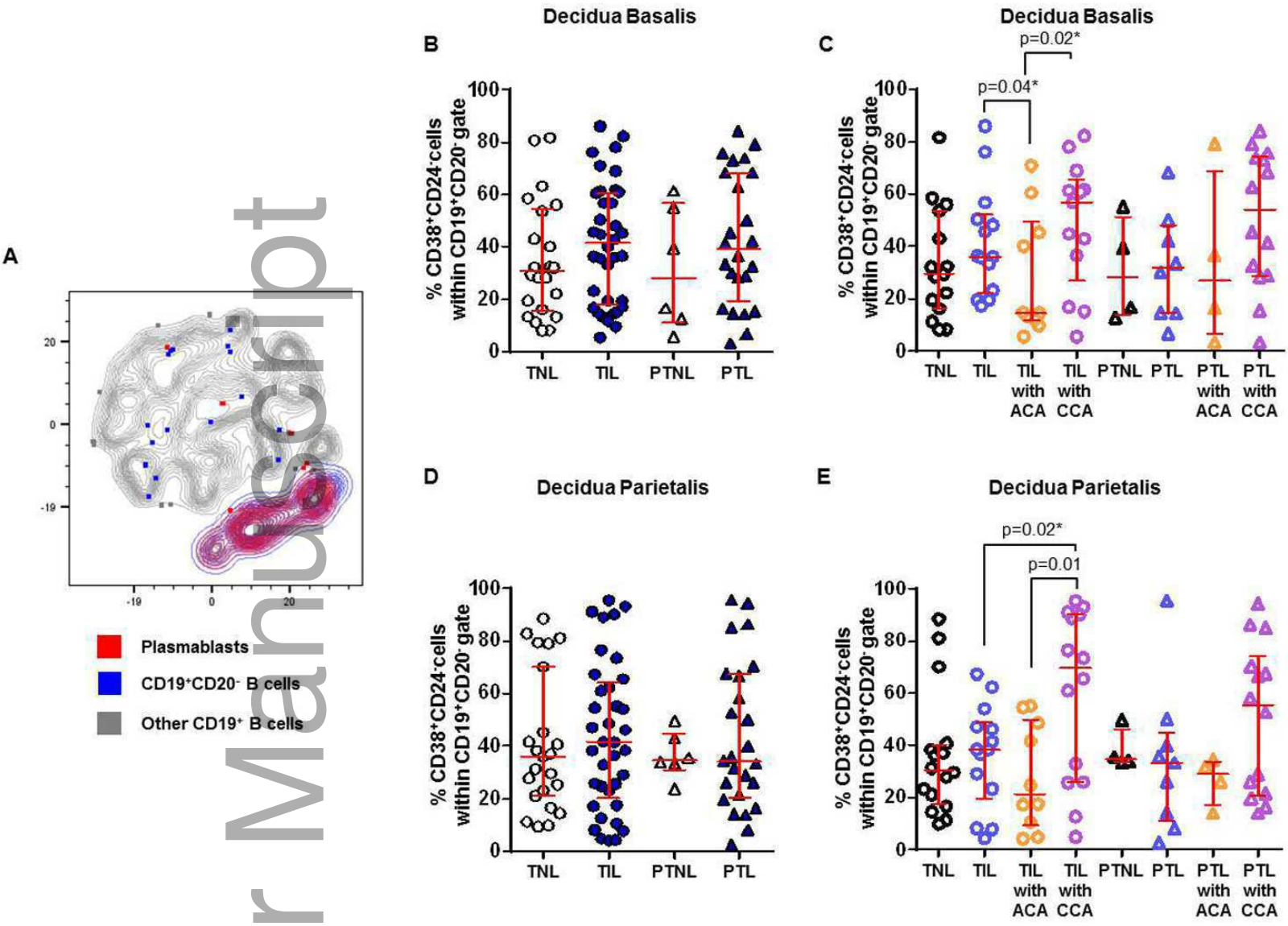

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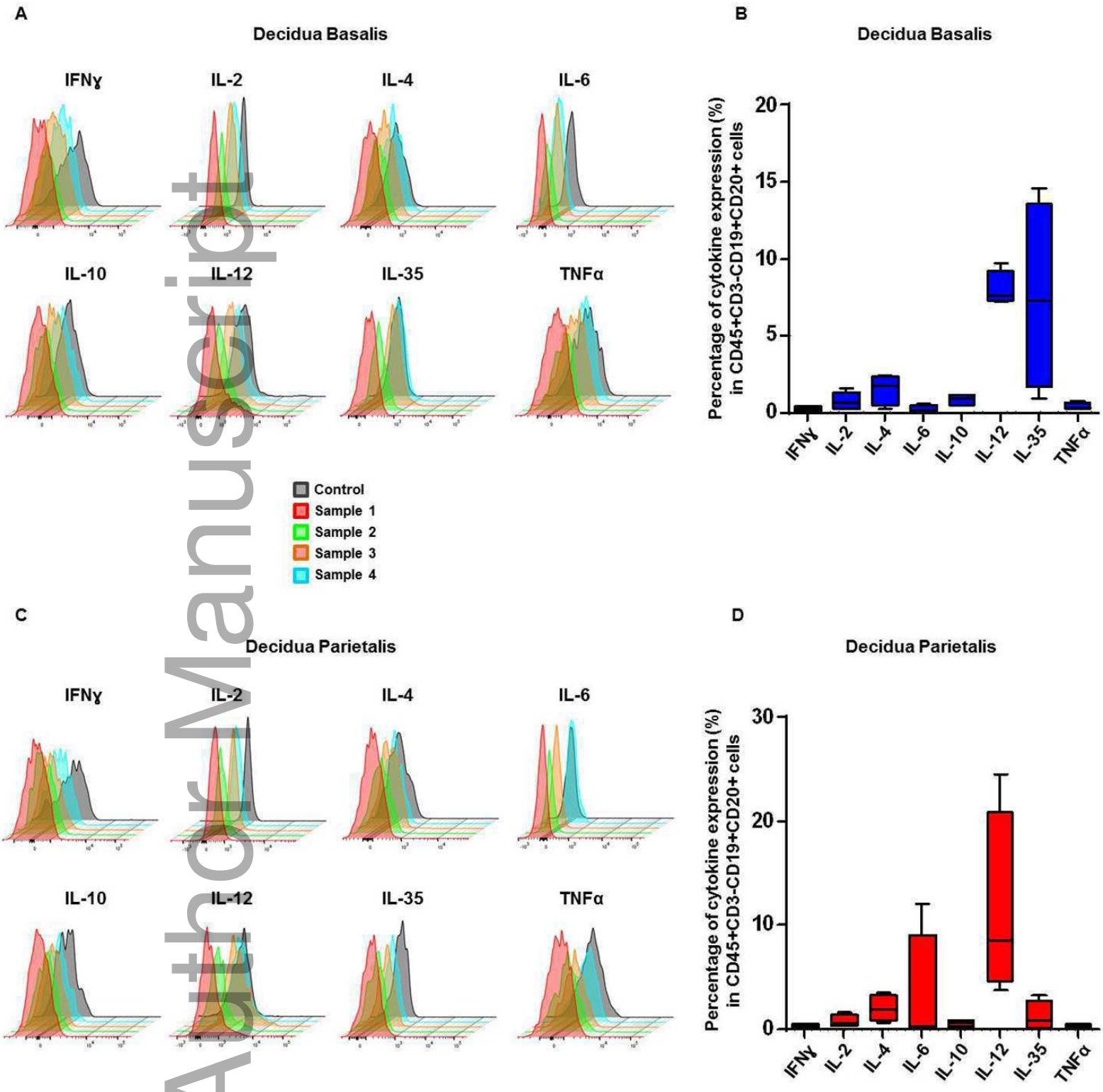

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