PREMATURE BIRTH AND CESAREAN SECTION AFFECT NEONATAL CD4$^+$ T CELL GENE EXPRESSION AND CELLULAR FUNCTION

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Abstract

Premature birth and cesarean section are major perinatal factors influencing immune development and are associated with increased morbidity and inflammatory diseases. However, their impact on neonatal immunity remains incompletely defined. To determine how gestational age and mode of delivery shape early immune programming, we analyzed CD4$^+$ T cells, central regulators of adaptive responses, from preterm neonates and full-term neonates born by cesarean section or natural birth.

We performed transcriptomic profiling (mRNA-seq) and functional assessment of T cell activation, proliferation, and cytokine production following stimulation. The mode of delivery exerted a dominant effect on the CD4$^+$ T cell transcriptome and function. CD4$^+$ T cells from full-term neonates delivered by natural birth exhibited an immune activation signature, produced higher levels of multiple cytokines, but showed reduced proliferative capacity.

In contrast, prematurity induced modest changes in basal gene expression relative to full-term cesarean section neonates. CD4+ T cells from preterm neonates displayed enhanced proliferation and increased secretion of inflammatory cytokines IL-13, TNFα, IL-6, and IL-17F upon stimulation, indicating heightened responsiveness.

Collectively, our findings show that $CD4^+$ T cells from preterm neonates exhibit augmented inflammatory capacity, which becomes more regulated at term. The mode of delivery further refines this developmental trajectory: cesarean section is associated with a restrained functional profile, whereas natural birth is associated with an immune activation signature and increased responsiveness. These results provide evidence that neonatal $CD4^+$ T cell trajectories are established during fetal life and subsequently modulated at birth, underscoring the layered influence of perinatal factors on immune development.

Keywords

$CD4^+$ T cells, premature birth, cesarean section, neonates, transcriptome

1. Introduction

The neonatal period, comprising the first 28 days of life, is the most vulnerable period for child survival. The neonatal immune system exhibits distinct characteristics and is believed to be programmed during the intrauterine period. This results in neonates having limited innate inflammatory and Th1 immune responses, but predominant Th2, Th17, and tolerant immune responses[1–5]. Neonatal T cells exhibit a limited activation response, biased toward innate immunity[6–9]. We previously published transcriptomic and epigenetic analyses of $CD4^+$[7] and $CD8^+$[6] T cells from human neonates, compared with adult naïve T cells, showing distinct functional programming characterized by high proliferation, a highly glycolytic metabolism, an innate-biased response, and low effector functions[10]. Furthermore, early events that occur during gestation, childbirth, and the perinatal period have a significant impact on an individual's development and health throughout life. The immune system´s development and response are also affected by early life events. Premature birth is one of

the major causes of death in early life, causing around 28% of deaths in the population of under-5-year-old children in Mexico[11,12]. In addition to the high rate of fatalities, premature babies often suffer morbidities that leave sequels that impact their health for life, particularly those who are born with a very low weight or before 33 weeks of gestational age (wga)[13,14]. Premature babies are highly susceptible to infections and have limited protective immune responses[2,15,16]. However, their immune system is hyperreactive, often leading to highly inflammatory conditions such as sepsis and necrotizing enterocolitis[17–20]. The causes of prematurity have been previously studied; maternal conditions such as preeclampsia and intrauterine infections have been associated, while maternal and fetal immune responses contribute to preterm labor[21–23].

The rate of cesarean section (c-section, cesarean) for babies born under 33 wga is between 45% and 72% worldwide[14]. In addition, many full-term babies are also delivered by cesarean section, often planned surgeries without a medical reason. Worldwide, the percentage of cesarean sections as compared to vaginal birth is 32.5% and has been on the rise[24]. However, in Mexico, 55% of total births and most premature babies are delivered by cesarean section, exceeding the world average[11,25].

The effects of prematurity and birth type on the development of the neonate and its immune response have been studied, but clear results remain lacking. Some epidemiological studies have shown, however, a predisposition of babies born by caesarean section to a series of metabolic and proinflammatory conditions, such as obesity, diabetes, asthma, and autoimmune diseases[25–29].

To better understand the impact of birth type and gestational age on the immune system, we evaluated $CD4^+$ T cells, as these cells are central to the strength and type of adaptive immune response. Here, we present the evaluation of the transcriptome, cell activation, proliferation, and cytokine secretion of $CD4^+$ T cells from preterm (28 to 36 weeks of gestation) newborns, as compared to full-term newborns born by cesarean section (cesarean) or natural birth (vaginal delivery). Our results show a greater difference in the transcriptome between neonates born by cesarean section and those born by natural birth, whereas prematurity exhibits only minor changes relative to controls. The gene expression

profile of natural birth $CD4^+$ T cells showed similarity with a mild "immune activation" signature. In our functional experiments, $CD4^+$ T cells from natural birth produce higher amounts of all cytokines but proliferate less than those from cesarean section. Of note, $CD4^+$ T cells from premature babies showed a remarkably high production of cytokines, especially IL-13, TNF-α, IL-6, and IL-17F, and a high proliferation rate as compared to cells from full-term neonates.

Together, these findings demonstrate that birth mode is a dominant determinant of neonatal $CD4^+$ T cell functional programming, whereas prematurity is associated with potent inflammatory responses. This study provides molecular and functional evidence that early-life immune trajectories are shaped before and at birth, underscoring the immunological relevance of delivery practices and gestational age in neonatal health.

2. Materials and Methods

Cell purification and stimulation

Neonatal blood was collected as previously described[7]. Briefly, the blood was collected just after the babies' birth by puncture of the umbilical cord vein, from births through vaginal delivery or cesarean section at Hospital General José G. Parres (Cuernavaca, Morelos, Mexico), Hospital General de Temixco (Temixco, Morelos, Mexico), Instituto Nacional de Perinatología (Mexico City, Mexico), with full informed consent from the mothers and the ethical approval of the hospital (CONBIOÉTICA-17-CEI-001-20160329). No samples were taken from mothers with any health conditions. An official agreement (File 1372/1706) was granted by Servicios de Salud Morelos to collect the blood samples in Cuernavaca and by the Instituto Nacional de Perinatología (2018-1-149). Samples were processed immediately to obtain mononuclear cells by density gradient centrifugation using Lymphoprep (Axis-Shield). Then, they were cultured overnight in tissue culture dishes (cat. 353003, Corning) to eliminate adherent cells and obtain mononuclear cells (CBMCs). To obtain $CD4^+$ T cells, CBMCs were mixed with the RosetteSep $CD4^+$ T-cell enrichment cocktail (STEMCELL) and 1 mL of cultured erythrocytes from the same blood sample, and then separated by a second density gradient. Cells were cultured at 37 °C and 5% CO_2 in supplemented RPMI medium

(10% Fetal bovine serum, 1% glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) (Thermo Fisher Scientific). All samples were at least 93% CD3+/CD4+, as determined by flow cytometry, and very similar purities were obtained in all populations (Supplementary figure 1). The purity of our populations of $CD4^+$ T cells was evaluated with antibodies (anti-CD4 (20-0048-T100) and anti-CD3 (20-0037-T100); TONBO biosciences). The absence of maternal cell contamination and the unstimulated status were verified by staining for CD45RO and CD69, markers of memory and activated T cells. Cells were stimulated by crosslinking both CD3 and CD28 antibodies (1 µg/mL) with a secondary antibody (Goat Anti-Mouse IgG Antibody (H + L) bs-0296G, Bioss).

RNA preparation and sequencing

We prepared total RNA using TRIzol reagent (Thermo Fisher Scientific, 15596-018, Invitrogen), as indicated by the manufacturer. RNA integrity was analyzed using a Bioanalyzer nano-RNA-Kit (Thermo Fisher Scientific), and only samples with an RNA Integrity Number (RIN) > 8.0 were included. For each sample, 500 ng of total RNA was used to prepare an RNA-seq library using the Illumina Stranded mRNA Prep Ligation kit, according to the manufacturer's instructions. The samples were sequenced on the NextSeq-500 Sequencer (Illumina). Sequencing was performed paired-end, with a real length of 75, and a sequencing depth of $47.04 \pm 11.3 \times 10^6$.

Transcriptome analysis

The workflow for transcriptome analysis was as previously described[7]. Briefly, it consisted in: i) evaluation of the Data quality (FASTQC program[30]); ii) elimination of adaptors and low-quality reads (Trimmomatic version 0.39)[31]; iii) reads alignment against the reference genome (GRCh38/hg38, using the STAR 2.4.2ª software[32]); iv) visualization of the alignment with IGV[33]; v) obtention of the read counts per gene (HTSeq-count)[34]; vi) Identification of the differentially expressed genes (DESeq2[35] package in R (Bioconductor), using an adjusted P value <0.05, and a |log fold change| ≥1); vii) identification of the enriched pathways (ClusterProfiler[36] package in R, using the function EnrichKEGG); viii) visualization of the enriched pathways (enrichplot (Bioconductor)). We also performed an analysis of transcription factor activity based on the expression of their targets using decoupleR[37].

Additionally, gene set enrichment analysis (GSEA)[38] was performed in R using the whole DESeq2 results table and the clusterProfiler package. Finally, a protein-protein interaction (PPI) network was generated for the differentially expressed genes (DEGs) with the STRINGdb[39] R package (v2.22.0), recovering all available interactions from the STRING database (v12.0) for Homo sapiens (taxonomy ID: 9606). Interactions were filtered using a minimum score of 0.7 (high confidence), and isolated nodes were removed from the final network. The network was visualized and analyzed in Cytoscape (v3.10.4)[40]. Functional enrichment analysis for Gene Ontology (GO) Biological Process terms was performed using the STRING enrichment function implemented in the stringApp plug-in for Cytoscape (v2.2.0)[41] to finalize network annotation.

Note: The anonymized datasets will be deposited in a public repository and made available upon publication.

Cell activation analysis

Cell response was measured by evaluation of i) the activation markers CD69 and CD25, ii) homeostatic and activation-induced proliferation, and iii) AP-1 and NF-κB activation. For the evaluation of activation markers, we used anti-CD69-FITC (cat. 310904, BioLegend) and anti-CD25-APC (cat. 20-0259-T100, Cytek). After 72 hours of stimulation, purified $CD4^+$ T cells were collected and incubated with a cell viability reagent (Invitrogen) to assess viability, as well as with anti-CD69-FITC and anti-CD25-APC for 30 minutes at 4°C. After incubation, cells were washed with PBS containing 2% FBS, then fixed with 1% formaldehyde.

To assess transcription factor activation, we evaluated p65 and c-Jun phosphorylation by intracellular staining. After cell stimulation, CBMCs were collected and washed with PBS containing 2% FBS, then washed again with 1X PBS. Subsequently, $CD4^+$ T cells were stained with anti-CD4-BV605 (cat. 317438, Biolegend) and a viability reagent (cat. L34975, Invitrogen), and incubated for 30 minutes at 4°C. After incubation, cells were washed with PBS supplemented with 2% FBS and then subjected to a permeabilization protocol, which included fixation with 1.5% formaldehyde for 10 minutes, permeabilization with cold absolute methanol for 10 minutes (0°C or −80°C), a wash with PBS supplemented with 2%

FBS, incubation with the antibody solution for 30 minutes at 4°C, and a final wash followed by fixation with 1% formaldehyde. Activation of p65 and c-Jun was assessed by evaluation of phosphorylation at specific residues. We used anti–phospho c-Jun (Ser63, cat. GTX61170, GeneTex), secondary anti–rabbit FITC-conjugated antibody (GeneTex), and anti–phospho p65 (Ser529)–APC (Miltenyi Biotec). Samples were acquired on an Attune NxT cytometer (Invitrogen), and data were analyzed using FlowJo software (Tree Star, CA). Differences in the levels of activated proteins between conditions were assessed using Mann–Whitney or Wilcoxon tests in GraphPad Prism 7.

Proliferation assay

CBMCs were left untreated or activated through the TCR and CD28 molecules for 96 h in the presence of CFSE (cat. 21888, Sigma) 50 nM. $CD4^+$ T cells were followed by staining the CD4 molecule (CD4-PerCP-Cyanine 5.5 (cat. 65-0048-T100, TONBO)). Cell division was evaluated by CFSE dilution using an Attune cytometer (Life Technologies) and analyzed with FlowJo X software.

Soluble cytokines/molecules

Production of cytokines and other soluble molecules by $CD4^+$ T cells (2×10^6 cells/mL) stimulated for 72h through the TCR and CD28 was evaluated using the Legendplex kit (cat. 741028, Biolegend), according to the manufacturer's instructions. The following cytokines were evaluated: Th1 (IFNγ and TNAα), Th2 (IL-13, IL-5, IL-6, and IL-4), Th9 (IL-9), Th17 (IL17A and IL17F), Th22 (IL-22), Treg (IL-10), and IL-2 with the standard Th kit. In addition, we evaluated innate cytokines (IL-7, IL-1β, and IL-8), TGF-β, and cytotoxic molecules (GZMB and Perforin) relevant to neonatal cells using an ad hoc kit. Cytokine quantification was performed by flow cytometry.

3. Results

The type of birth is an important determinant of the gene expression profile

To investigate the impact of preterm birth and cesarean section on the gene expression profile of $CD4^+$ T cells, we obtained the transcriptome of $CD4^+$ T cells from preterm neonates

born by cesarean section and full-term newborns born by cesarean section or natural birth (vaginal delivery). First, we purified cells from preterm newborns (28 to 33 wga) born by cesarean section (preterm, PT) and full-term babies (37 to 41 wga) born by cesarean section as controls (cesarean, CS). Additionally, we purified cells from neonates born full-term via vaginal delivery (natural birth, NB) to compare them with those from term cesarean section. To ensure cell purity and prevent cross-contamination with maternal blood, we evaluated CD45RO and CD69 expression in each sample by flow cytometry. As shown in Supplementary Figure 1, all cells showed comparable purity across populations. All samples had CD3+CD4+ purity above 93% and were negative for $CD45RO^{hi}$ or CD69 expression.

The transcriptome (Supplementary Tables 1-4) was analyzed as previously described[7]. As an indication of the dispersion of the data, we show a principal components graph (Fig. 1A). To our surprise, the biggest separation among the three different $CD4^+$ T cell populations was found between $CD4^+$ T cells obtained from neonates born at term by cesarean section (CS) and those of natural birth (NB). Preterm (PT) samples were dispersed, perhaps because the ages varied between 28 and 33 wga. Preterm and full-term babies born by cesarean section showed no real separation in the PCA. We present a volcano plot of differentially expressed genes between natural birth and cesarean section samples, showing 600 differentially expressed genes (Fig. 1B). Some of the genes overexpressed by the natural birth cells were *IL1B, TNFA, CD40, LTBR, CD36, FOSB,* and *FGR*, which are associated with immune responses (Figure 1B). On the other hand, cells from cesarean sections expressed KLF9, MIR186, and MIR1244-4, all of which are associated with the regulation of proliferation/differentiation. We used the overexpressed genes from these two populations to perform overrepresentation analysis with clusterProfiler[36]. Only one pathway, taste transduction, was found enriched in the full-term cesarean section samples as compared to natural birth samples. These are genes from G protein signaling pathways with innate immune functions[42,43]. In the natural birth samples, however, 27 pathways were found enriched (Supplementary Fig. 2B). The most significant pathways and their corresponding genes are shown in Figure 1C-D. These pathways are related to cytokine and chemokine expression, as well as their receptors, particularly those associated with inflammatory pathways such

as TNF and IL-1; T cell activation, signal transduction (CD40, ICAM1, MPK, and FOSB), and cell motility (amoebiasis). Next, we identified differentially expressed genes between $CD4^+$ T cells from preterm (PT) and full-term (cesarean section, CS) neonates (Supplementary Fig. 2A). Only 16 genes were found differentially expressed, with the overexpression of CLIC3, a chlorine channel involved in pH, volume, and cell growth regulation, PTPRN2, a phosphatase involved in the secretory pathway, and PTGDR2 involved in prostaglandin metabolism. Overrepresentation analysis didn't identify any pathways enriched with these DEGs. All the DEGs from the 2 comparisons are shown in Supplementary Fig. 3.

We also performed gene set enrichment analysis to further identify enriched processes and pathways. In the comparison between NB and CS samples, we identified Biological Processes and Pathways related to adaptive immune response, effector response, chemotaxis, and cytokine production in natural birth $CD4^+$ T cells, while CS transcriptomes were enriched for pathways of RNA processing and splicing (Fig. 2A-B). The protein-protein interaction network generated with these genes is shown in Fig. 2E, which shows two main subnetworks: the bigger one related to immune response (*TNF, CD36, CD40, ICAM1, NKG7, GNLY, IL-1β, NLRP3, EOMES, TBX21, PTGS2, BCL6,* and others) and the smaller one related to regulation of cell cycle (*FOXM1, CDC45, MCM10, CDC20, TICRR, AURKB,* and others). When comparing PT and CS samples, a low oxygen response was observed in the PT samples (Fig. 2C-D). In the analysis, there was a notable enrichment of pathways related to proliferation, glycolysis, aminoacid biosynthesis, and the cytoskeleton in cells from the premature samples.

We also performed Transcription Factor Activity analysis with decoupleR[37]. In Figure 3, we show the results of the transcription factor (TF) activity. When comparing NB and CS samples, we identified NF-κB, AP-1, STAT1, 3, 6, 5A, HIF1A, and MYC activity in CD4+ T cells from natural birth, which are typically associated with inflammatory responses.

These results contrast with our previous report, which showed that, compared with adult $CD4^+$ T cells, neonatal cells from natural birth exhibit a gene expression profile characterized by a limited immune response[7]. However, compared with CS cells, CD4+ T cells from NB exhibit an immune activation signature, presumably due to brief activation associated with

vaginal delivery, though not reaching adult levels. On the other hand, the transcription factor analysis didn't identify statistically significant hits in the comparison between CS and PT samples.

Taken together, these results indicate that $CD4^+$ T cells from natural birth show a mild "immune activation" signature, while cesarean section cells lack this immune signature.

Activation of CD4+ T cells is influenced by the type of birth

We next sought to determine whether differences existed in the responsiveness of the three $CD4^+$ T cell populations to TCR/CD28 stimulation. Therefore, we stimulated $CD4^+$ T cells from full-term neonates born by natural birth or cesarean section or preterm babies *in vitro*, crosslinking soluble antibodies (CD3/CD28) with an anti-mouse antibody for 72h, and evaluated the expression of the activation markers CD69 and CD25. As shown in Fig. 4A-B, $CD4^+$ T cells from all three populations were similarly activated. Interestingly, CD25 expression was higher (23-51%) than CD69 expression (5-13%) in all three populations at that time point (Fig. 4B).

We also evaluated the phosphorylation of two important transcription factors as a measure of their activation upon TCR/CD28 signals, p-ser529-p65 (NF-kB)[44] and p-ser63-c-Jun (AP-1)[45], as previously reported[46]. The gating strategy for these evaluations is shown in Supplementary Fig. 4A. All three populations showed p65 activation (phosphorylation) upon stimulation (Fig. 4C and E), whereas preterm cells exhibited lower MFI levels (Fig. 4C). On the other hand, a higher induction of c-Jun activation (phosphorylation) was observed in the samples of natural birth as compared to both term and preterm neonates born by cesarean section (Fig. 4D-F). However, the cesarean section term $CD4^+$ T cells did show activation, while preterm cells showed lower activation of c-Jun (Fig. 4F). This is in agreement with the higher expression of MAPKs and c-Fos in the natural birth samples in the transcriptomic analysis (Fig. 1D). Of note, it has been reported that heightened Calcium signaling, but lower NFAT activation and NFAT-mediated transcription, occur in neonatal T cells[9,47]. Also, it has been described that activation of NFAT (calcium signals) without proper activation of AP-1 signals can lead to the activation of the Th2 cytokine profile, characterized

by a higher expression of IL-4 and IL-13, and predisposing to allergies and asthma, as has been observed in the cesarean section-born neonates[48].

T cells from preterm and full-term neonates born by cesarean section show high proliferation rates

We evaluated the proliferation of the three $CD4^+$ T cell populations in response to activation by soluble antibodies (CD3/CD28). The gating strategy used for this is shown in Supplementary Fig. 4B. In response to stimulation by TCR/CD28 signals, all the populations proliferated for up to three or four generations (Fig. 5A). A higher percentage of cell division was observed in $CD4^+$ T cells from cesarean section babies, both full-term and preterm (Fig. 5B). This is consistent with the gene expression profile and functional annotation of preterm $CD4^+$ T cells, which indicate cell cycle progression. This high activation-induced proliferation in preterm and cesarean cells contrasts with the results for transcription factor activation, particularly c-Jun, which was more pronounced in natural birth $CD4^+$ T cells but did not reach the same levels in preterm cells.

$CD4^+$ T cells produce cytokines from all Th phenotypes when stimulated in isolation

To evaluate the functional properties of the three neonatal $CD4^+$ T cell subsets, cells were stimulated through TCR/CD28 for 72 h. Cytokines representative of major T helper (Th) lineages were then quantified in culture supernatants using a LegendPlex multiplex assay and analyzed by flow cytometry. Upon TCR/CD28 stimulation, $CD4^+$ T cells from all three populations produced IL-2 (Fig. 6). However, average production was highest in preterm cells, followed by those from natural birth, and only a small production was detected in cesarean section cells.

$CD4^+$ T cells from natural birth produced a significant amount of all four Th2 cytokines (IL-4, IL-5, IL-6, and IL-13). Cells obtained from the full-term cesarean section neonates only induced a small amount of IL-13. On the other hand, cells from preterm neonates showed higher expression of all Th2 cytokines, with markedly elevated IL-13 production.

The evaluation of Th1 cytokines showed, as expected, no significant induction of IFN-γ upon stimulation, yet, within the low amount of this cytokine, a higher level was observed in the cells of natural birth neonates compared with those from cesarean section. Again, a high degree of dispersion was observed in preterm neonates, with some samples showing higher IFN-γ levels. TNF-α expression was observed in full-term babies, with lower levels in cesarean section neonates. The cells of the preterm babies showed a very high expression of this cytokine.

In the Th17 profile, only IL-17F showed a significant induction by TCR/CD28 signals in full-term neonates. The expression of this cytokine was significantly higher in preterm cells than in term cells from cesarean section neonates.

IL-10 was expressed in all stimulated cells, with a tendency to higher expression in preterm cells, while TGF-β1 was not detected. IL-9 expression was very low in all samples.

On the other hand, IL-22 was induced by TCR/CD28 signals in all cell populations, but was higher in preterm babies than in cesarean section neonates.

Other molecules relevant to neonatal cell biology were also evaluated, like innate cytokines (IL-1β and IL-8), IL-7, and cytotoxic molecules (GZMB and PRF), which are shown in Fig. 7. IL-7 was not produced in any samples, while only two samples of pre-term babies induced the expression of Il-1β. The chemokine IL-8 was stimulated in all three cell populations of neonatal T cells, as was previously described[49]. Interestingly, Granzyme B expression was stimulated by TCR/CD28 signals, whereas perforin expression remained stable across conditions. The biological significance of this expression in neonatal $CD4^+$ T cells is yet to be determined.

When stimulated in the presence of other mononuclear cells (CBMCs), T cells express a more restricted panel of cytokines and molecules (Supplementary Fig. 5). Only IL-6, IL-10, IL-1β, and IL-8 are produced in all three populations, as well as Granzyme B and perforin. However, IL-1β is more produced in natural birth CBMCs, which also produce IL-5. On the other hand, in cells from preterm babies, IL-5, IL-13, IL-17F, and IL-22 are also significantly produced. Besides IL-8, which is the most highly expressed across all three populations, IL-6, IL-13, IL-17F, and Granzyme B are the most highly expressed in preterm CBMCs. The

mechanism underlying these discrepancies is unclear, but it may be due to the direct or indirect roles of other cells present in the CBMC culture, or to the contribution of $CD8^+$ T cells.

4. Discussion

The transcriptomic results identified a functional programming of $CD4^+$ T cells from natural birth (NB) compared with cesarean section (CS) in term neonates. The gene expression profile was enriched for pathways and processes associated with immune responses, including T cell activation, cytokine pathways (TNF, IL1, and IL17), and Integrin and adhesion molecule signaling. We call this feature of NB CD4+ T cells "natural birth-associated immune activation" signature, which is associated with their greater response to TCR/CD28 stimulation *in vitro*. However, it remains unclear whether this immune activation is an effect of parturition, occurs before labor, is an indicator of neonatal T cells' participation in labor induction, or all of the above. One possible hypothesis is bystander activation of $CD4^+$ T cells during labor, driven by signals that promote parturition, such as maternal cytokines[50,51], stress molecules due to passage through the birth canal, or longer-term changes in oxygen levels.

Following *in vitro* stimulation with soluble antibodies (CD3/CD28), $CD4^+$ T cells from all three populations showed similar levels of activation markers (CD25 and CD69) at 72h. Notably, CD69 levels were low (5-13%) in neonatal T cells, while CD25 was more expressed (25-51%) in all three populations upon stimulation. This indicates that typical T cell activation markers may not function similarly in neonatal cells. Additionally, we evaluated p65 (NF-κB) and c-Jun (AP-1) phosphorylation as a proxy of their activation. Higher activation (phosphorylation) of c-Jun and p65 was observed in NB and CS samples as compared to PT ones. This could affect the gene expression profile of PT CD4+ T cells, including genes that require AP-1 or NF-κB activation. Of note, it has been previously reported that neonatal NB $CD4^+$ T cells have diminished AP-1-dependent transcription when compared to their adult counterparts[9]. Notably, it has been reported that NFACTc2 expression and NFAT activation in neonatal T cells are also diminished relative to those in adult naïve cells[47,52,53].

Regarding cell proliferation in response to stimulation (TCR/CD28 signals), a higher percentage of cell division was observed in $CD4^+$ T cells from CS babies, both full-term and preterm (Fig. 5), as compared to NB cells. This is very much in contrast to the activation of c-Jun, which is higher in NB samples.

Upon stimulation, purified $CD4^+$ T cells from NB produced small but significant amounts of cytokines from phenotypes Th1, Th2, Th17, Th22, Treg, IL-8, IL-2, and cytotoxic molecules granzyme B and perforin. However, when stimulated in the CBMC mixture, T cells only produced IL-5, IL-6, IL-1β, IL-8, granzyme B, and perforin. This suggests that full-term neonatal CD4+ T cells from NB have strong immune potential, which may be modulated by their regulatory environment, either through soluble or direct factors. Another confounding factor could be the reactivity of $CD8^+$ T cells in these mixtures. On the other hand, $CD4^+$ T cells from term CS neonates produce very low levels of only a few cytokines, with detectable levels of IL-2, IL-13, TNF-α, IL-17F, IL-10, and IL-22, as well as normal levels of IL-8, granzyme B, and perforin. Of note, CS $CD4^+$ T cells produce fewer IFN-γ, TNF-α, IL-1β, and perforin than NB cells. In the CBMCs mixture, CS cells produce only IL-10, IL-6, IL-1β, IL-8, granzyme, and perforin. This might indicate that CS cells are blocked from a Th1 response, while NB cells are more prone to respond in this way, which correlates with T-bet (TBX21) expression in NB cells. This is in agreement with the previously reported predisposition of cesarean section newborns to asthma and allergies[28,54].

Preterm (PT) cesarean $CD4^+$ T cells express a variable quantity of cytokines, with a high average. Due to dispersion, not all data were significant; however, cytokines IL-2, IL-6, IL-17F, TNF-α, and IL-8, as well as the molecules granzyme B and perforin, were produced at high levels by these cells in isolation. This is consistent with previous reports associating IL-6[55], TNF-α[56] and IL-8[57] in preterm $CD4^+$ T cells. Of note, IL-6, IL-13, IL-17F, IL-22, granzyme B, and perforin were produced significantly higher than in term CS $CD4^+$ T cells. In the culture of CBMCs, PT T cells produce IL-5, IL-6, IL-13, IL-17F, IL-10, IL-22, IL-1β, IL-8, granzyme B, and perforin. Of those, IL-5 and IL-22 were produced in higher amounts than in CS term T cells. This result confirms that preterm $CD4^+$ T cells are highly inflammatory, as previously suggested[55,58]; however, more cytokines and molecules might be relevant in the

pathophysiology of preterm labor than previously recognized, and special attention should be paid to the relevance of IL-13, TNF-α, and IL-17F in the immune pathologies of the preterm neonates.

Together, our findings suggest that neonatal $CD4^+$ T cells acquire the capacity to mount inflammatory responses in utero, as evidenced by the heightened functional profile observed in preterm neonates. By term, these cells appear to transition toward a more regulated or tolerogenic state, with reduced responsiveness, as evidenced by samples obtained from cesarean section deliveries. In contrast, the physiological processes associated with vaginal birth appear to actively reprogram $CD4^+$ T cells, promoting a more complex and functionally balanced immune profile, which is responsible for the "immune activation" profile. This natural birth-associated reprogramming likely reflects exposure to perinatal environmental cues that fine-tune $CD4^+$ T cell responsiveness within the broader cellular and tissue context.

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Authorship contribution statement

CJVM and LAKC participated in the investigation and formal analysis, wrote the original manuscript, and revised the final version. SMG, ACB, IYCC, and SVR participated in the

investigation. ACHR, CI, and SS revised the manuscript. AS participated in the conceptualization of the idea, oversaw the project, wrote the original manuscript, edited, and revised the final version. ORJ conceptualized the idea, oversaw the project, wrote the original manuscript, edited and revised the final version.

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Figure legends

Figure 1. Transcriptomic profiling of CD4$^+$ T cells from neonates born at term by natural birth and cesarean section, or preterm neonates. (A) Principal component analysis of CD4+ T cells from neonates born by natural birth (NB) or cesarean section (CS) at term, and preterm (PT). **(B)** Volcano plot showing the differentially expressed genes between natural birth and cesarean section samples. **(C)** Pathway enrichment by overrepresentation analysis with clusterProfiler between natural birth and cesarean section samples. (D) Network of genes and pathways of the natural birth condition.

Figure 2. Gene set enrichment analysis and protein–protein interaction (PPI) network. Gene set enrichment analysis of CD4+ T cells from neonates born by natural birth (NB) or cesarean section (CS) using the GO Biological Processes **(A)** and KEGG **(B)** databases. Gene set enrichment analysis of CD4+ T cells from neonates born at term by cesarean section (CS) or preterm, using the GO Biological Processes **(C)** and KEGG **(D)** databases. Protein–protein interaction (PPI) network of differentially expressed genes in CD4+ T cells from NB and CS neonates constructed using the STRING database and annotated with the most representative GO Biological Processes terms **(E)**.

Figure 3. Transcription factor activity in neonatal CD4+ T cells. (A) Heatmap showing inferred transcription factor activity scores (z-scores) for selected transcription factors across individual samples from neonates born by natural birth (NB), cesarean section (CS),

and preterm birth (PT). Colors indicate relative activity levels, ranging from low (blue) to high (red). Samples and transcription factors were organized using unsupervised hierarchical clustering. **(B)** Comparative analysis of transcription factor activity between natural birth and cesarean section. Bars represent differential activity scores, ordered from lowest to highest, with color indicating the associated *p-value*. **(C)** Comparative analysis of transcription factor activity between preterm birth and cesarean section, displayed analogously to panel B.

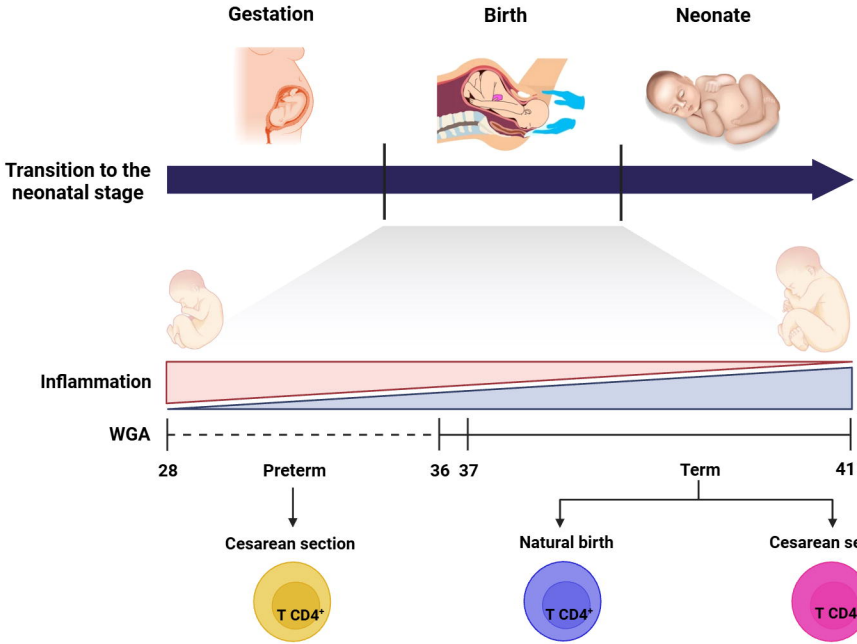
Figure 4. Evaluation of CD69 and CD25 markers and transcription factors activation. (A) We assessed T cell activation by measuring the expression of CD69 and CD25 on the cell surface. Purified $CD4^+$ T cells from term and preterm neonates were either left unstimulated or stimulated under the indicated conditions for 72 hours. The percentages of $CD69^+$ and $CD25^+$ T cells were determined by flow cytometry. **(B)** For transcription factor activation, we evaluate phosphorylation of c-Jun and p65 as markers of AP-1 and NF-κB activation, respectively. CBMCs were either left unstimulated or stimulated under the indicated conditions for 90 minutes (to measure c-Jun activation) or 60 minutes (for p65 activation). Mean fluorescence intensity (MFI) values for the indicated phosphoproteins were determined by flow cytometry. **(C)** MFI normalized values. The Wilcoxon test was used for paired samples, and the Mann–Whitney U test was used for unpaired samples. *P* values are shown above the error bars. $*P < 0.05$, $**P < 0.01$. For the evaluation of CD69 and CD25 expression, the number of samples was as follows: vaginal delivery = 13, cesarean section = 9, and preterm birth = 3. For the evaluation of transcription factor activation, the sample sizes were: Natural birth = 7, Cesarean = 8, and Preterm = 5.

Figure 5. Proliferation assay. CBMCs from term and preterm neonates were stained with CFSE and either left unstimulated or stimulated by crosslinking CD3 and CD28 molecules for 96 hours, followed by staining with anti-CD4–PerCP-Cy5.5. **(A)** Representative histograms of CFSE dilution in vaginal delivery, cesarean section, and preterm birth samples; **(B)** Percentage of division in unstimulated or stimulated CD4+ T cells. Statistical comparisons

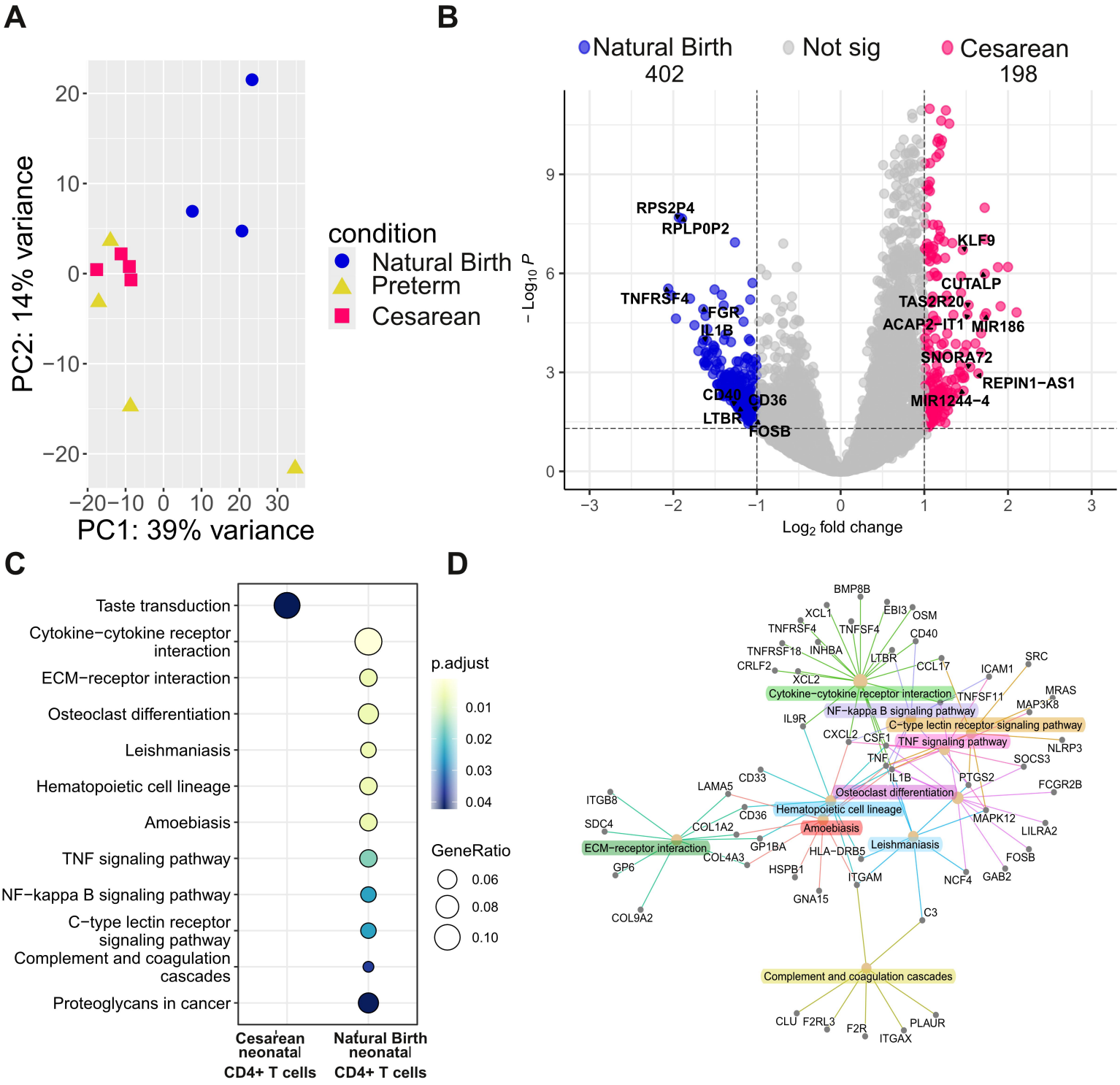
were performed using the Wilcoxon test for paired samples and the Mann–Whitney U test for unpaired samples. *$P < 0.05$. The sample sizes were as follows: Natural birth = 5, Cesarean = 6, Preterm = 5.

Figure 6. T helper cytokines production. Purified $CD4^+$ T cells from term and preterm neonates were either left unstimulated or stimulated by crosslinking CD3 and CD28 molecules for 72 hours. After incubation, supernatants were collected, and cytokine levels were measured using the LEGENDplex™ with a T Helper Panel. Cytokine concentrations were used for statistical analysis using the Wilcoxon test for paired samples and the Mann–Whitney U test for unpaired samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The number of samples was Natural birth = 16, Cesarean = 7, and Preterm = 4.

Figure 7. Innate cytokines and cytotoxic molecules production. Purified $CD4^+$ T cells from term and preterm neonates were either left unstimulated or stimulated by crosslinking CD3 and CD28 molecules for 72 hours. After incubation, supernatants were collected, and cytokine levels were measured using the LEGENDplex™ with a designed panel (IL-7, IL-1β, IL-8, Granzyme B, and Perforin). Concentrations were used for statistical analysis using the Wilcoxon test for paired samples and the Mann–Whitney U test for unpaired samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The number of samples was Natural birth = 13, Cesarean = 6, and Preterm = 4.



	Preterm	Term	
	Cesarean section	Natural birth	Cesarean section
CD4⁺ T cell response	• **High production of IL-2, TNF-α, Th2, Th17, Th22, Treg cytokines and GZMB, PRF.** • **Low NF-κB activation.** • **High proliferation.** • **Enriched pathways**: Low oxygen response and glycolysis.	• **Low production of IL-2, TNF-α, Th2, Th17, Th22, Treg, IL-8 cytokines and GZMB, PRF.** • **NF-κB and AP-1 activation.** • **Medium proliferation.** • **Enriched pathways**: Effector response, cytokine production, chemotaxis and glycolysis.	• **Detectable production of IL-2, TNF-α, IL-13, Th17, Th22, Treg, IL-8 cytokines and GZMB, PRF.** • **NF-κB and AP-1 activation** • **High proliferation** • **Enriched pathways**: RNA processing and splicing.
Cytokines produced in presence of other cells (CBMC)	**Th2, Th17, Th22, Treg, Innate and GZMB, PRF.**	**IL-5, IL-6, Treg, Innate and GZMB, PRF.**	**IL-6, IL-1β, Treg and GZMB, PRF.**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PC1: 39% variance
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TNFRSF4
FGR
IL1B
CD40
CD36
LTBR
FOSB
KLF9
CUTALP
TAS2R20
ACAP2-IT1
MIR186
SNORA72
REPIN1-AS1
MIR1244-4
Log2 fold change
C
Taste transduction
Cytokine-cytokine receptor interaction
ECM-receptor interaction
Osteoclast differentiation
Leishmaniasis
Hematopoietic cell lineage
Amoebiasis
TNF signaling pathway
NF-kappa B signaling pathway
C-type lectin receptor signaling pathway
Complement and coagulation cascades
Proteoglycans in cancer
Cesarean neonatal CD4+ T cells
Natural Birth neonatal CD4+ T cells
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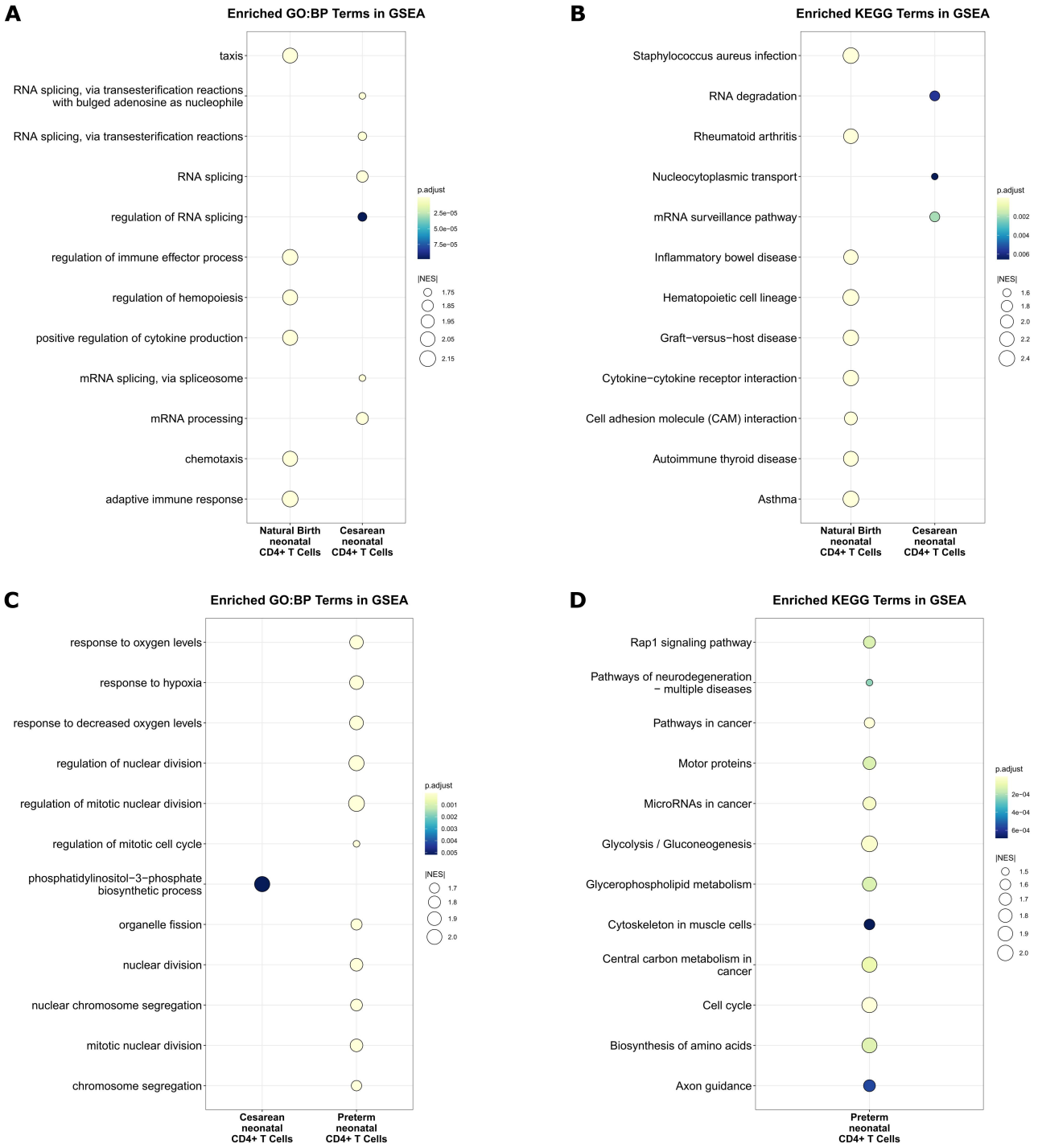
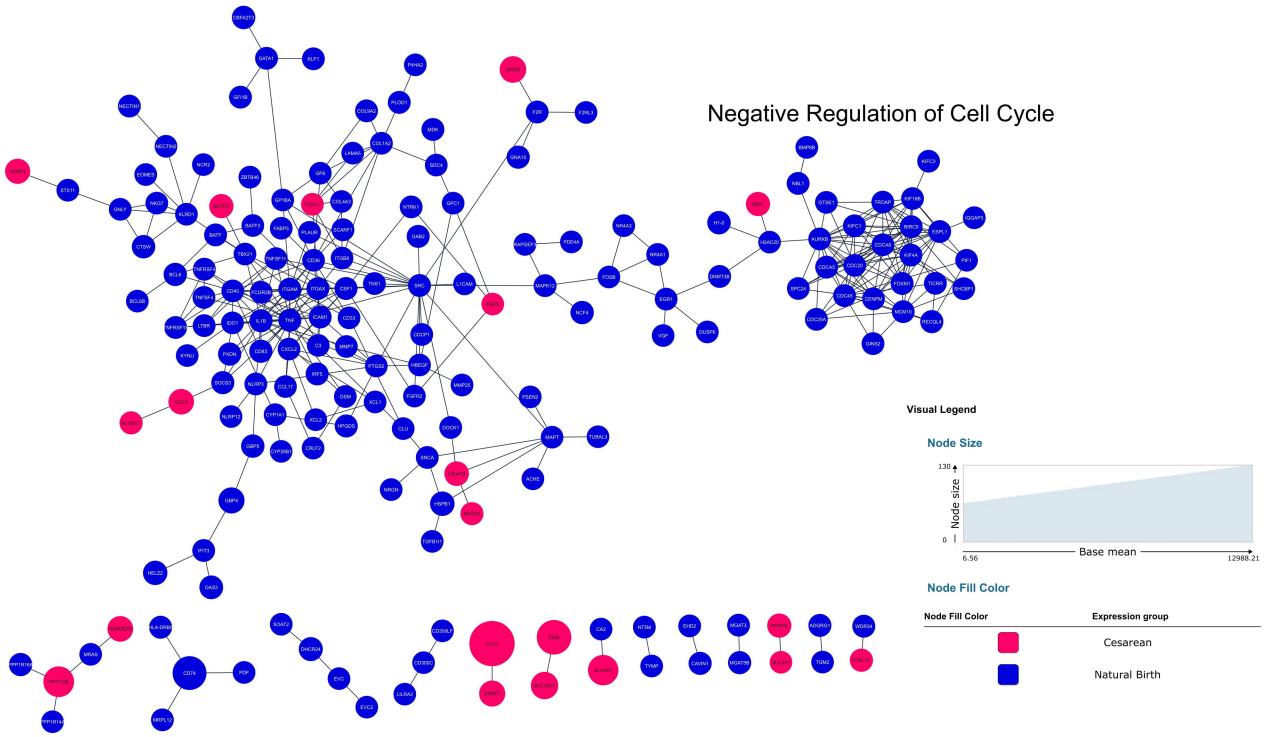
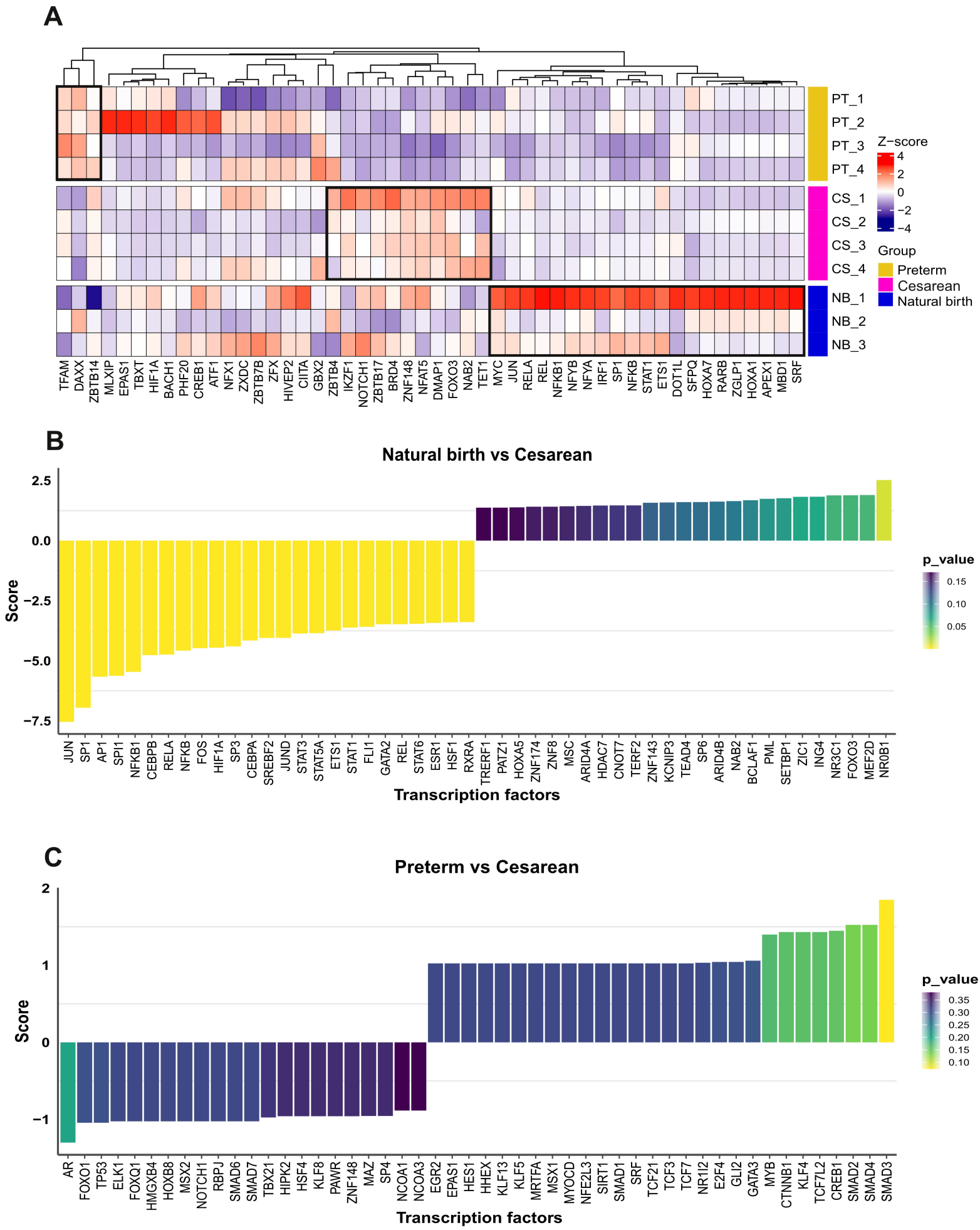


E Immune effector process / Regulation of innate immune response

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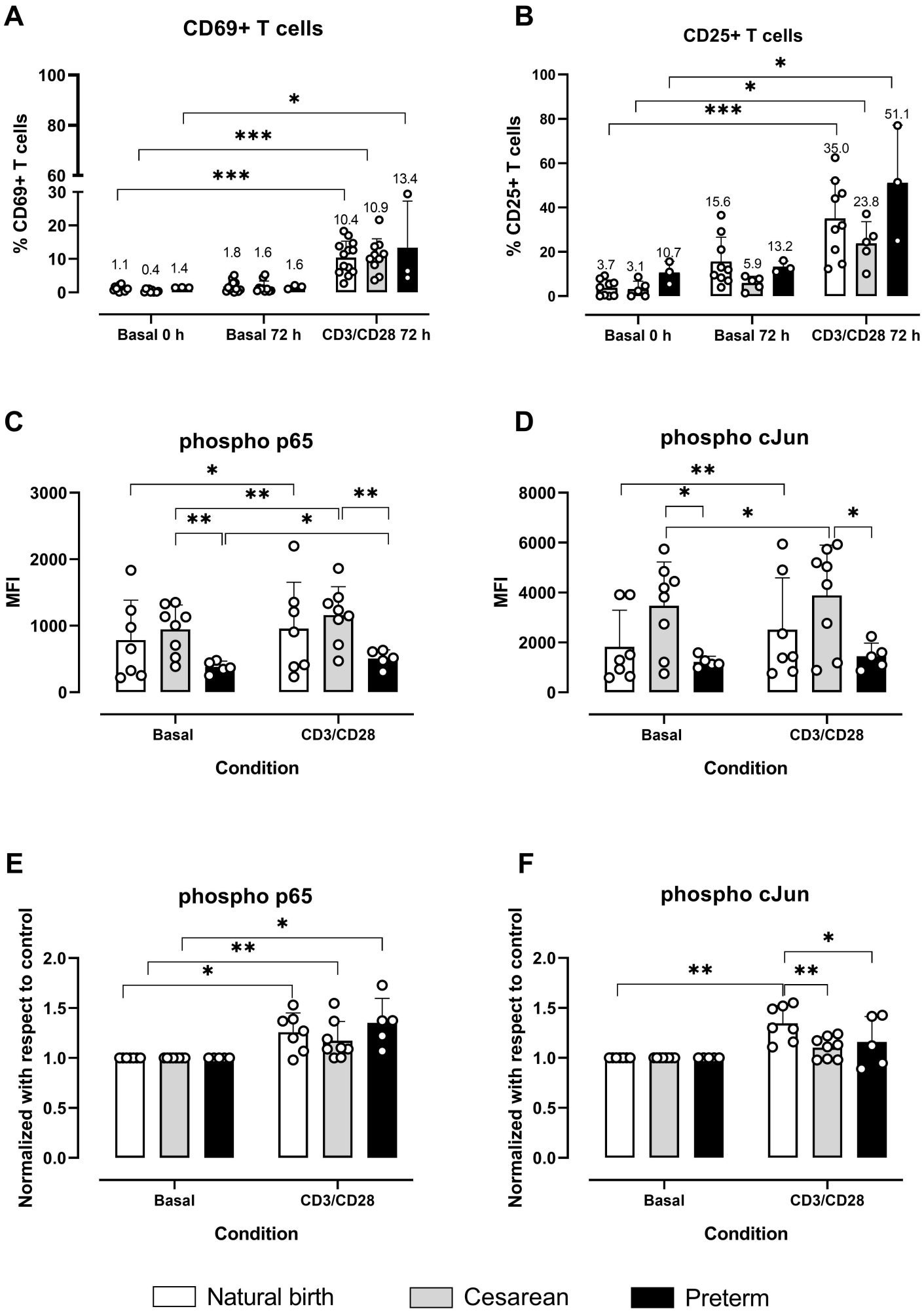
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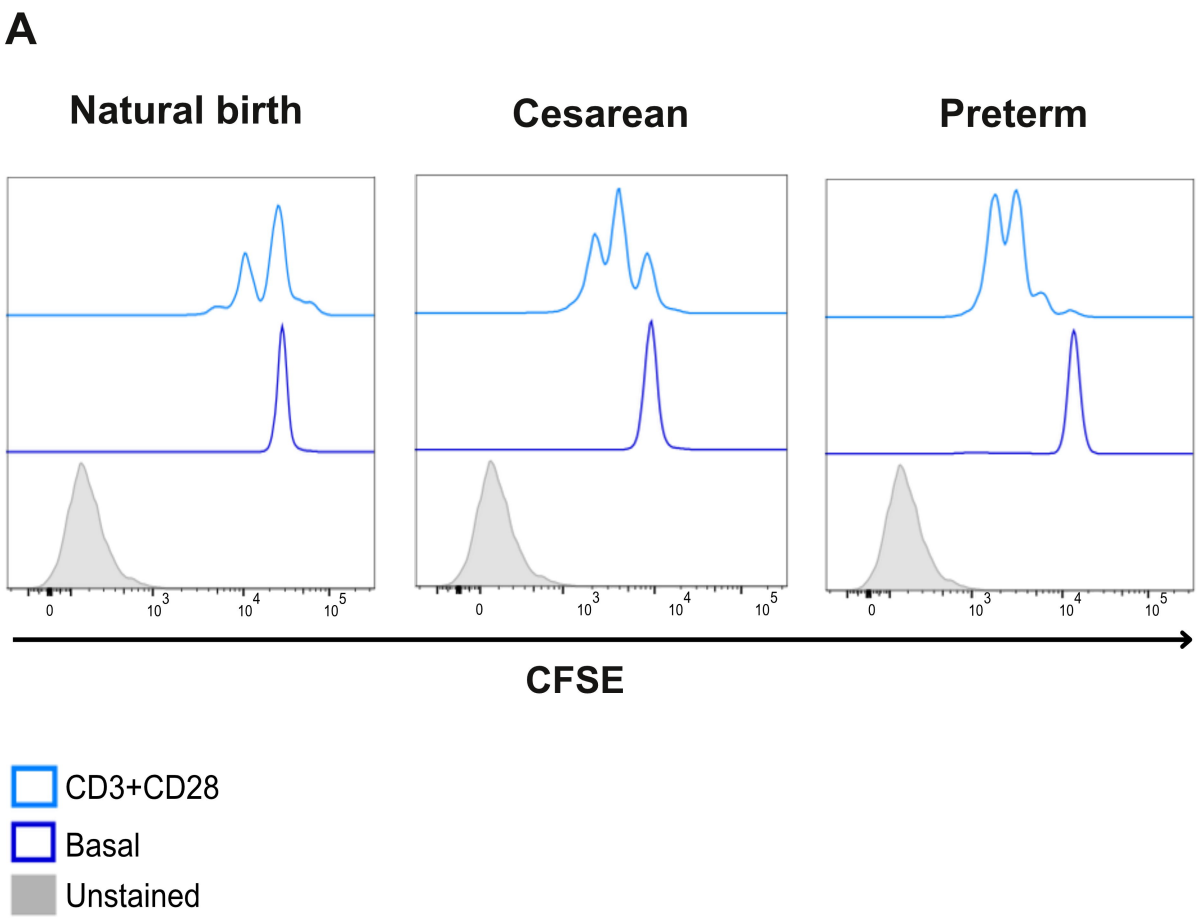
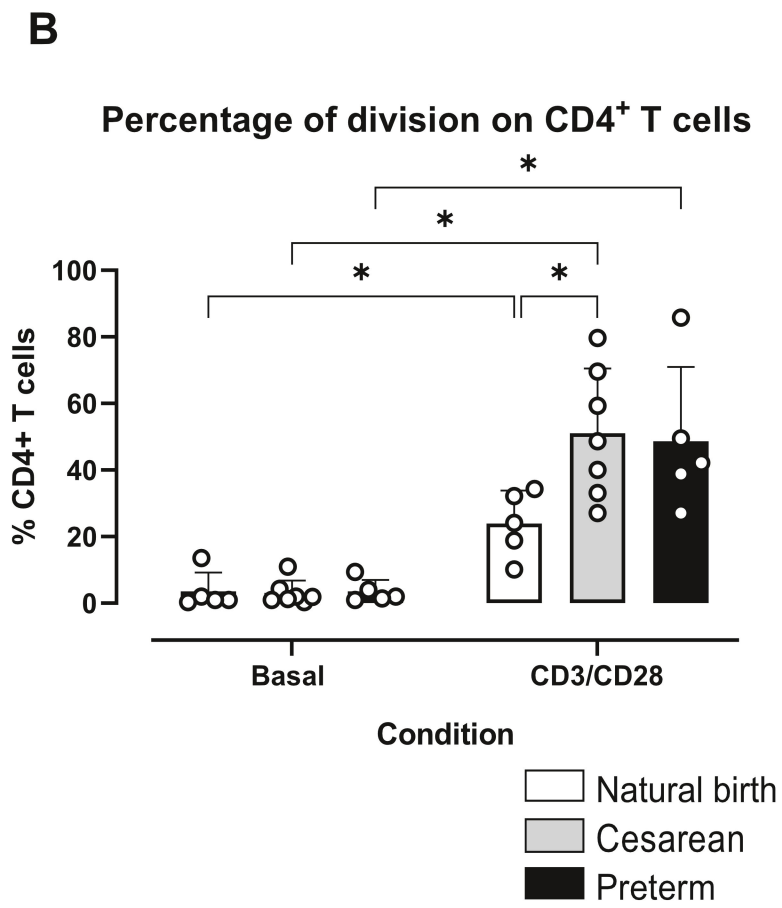
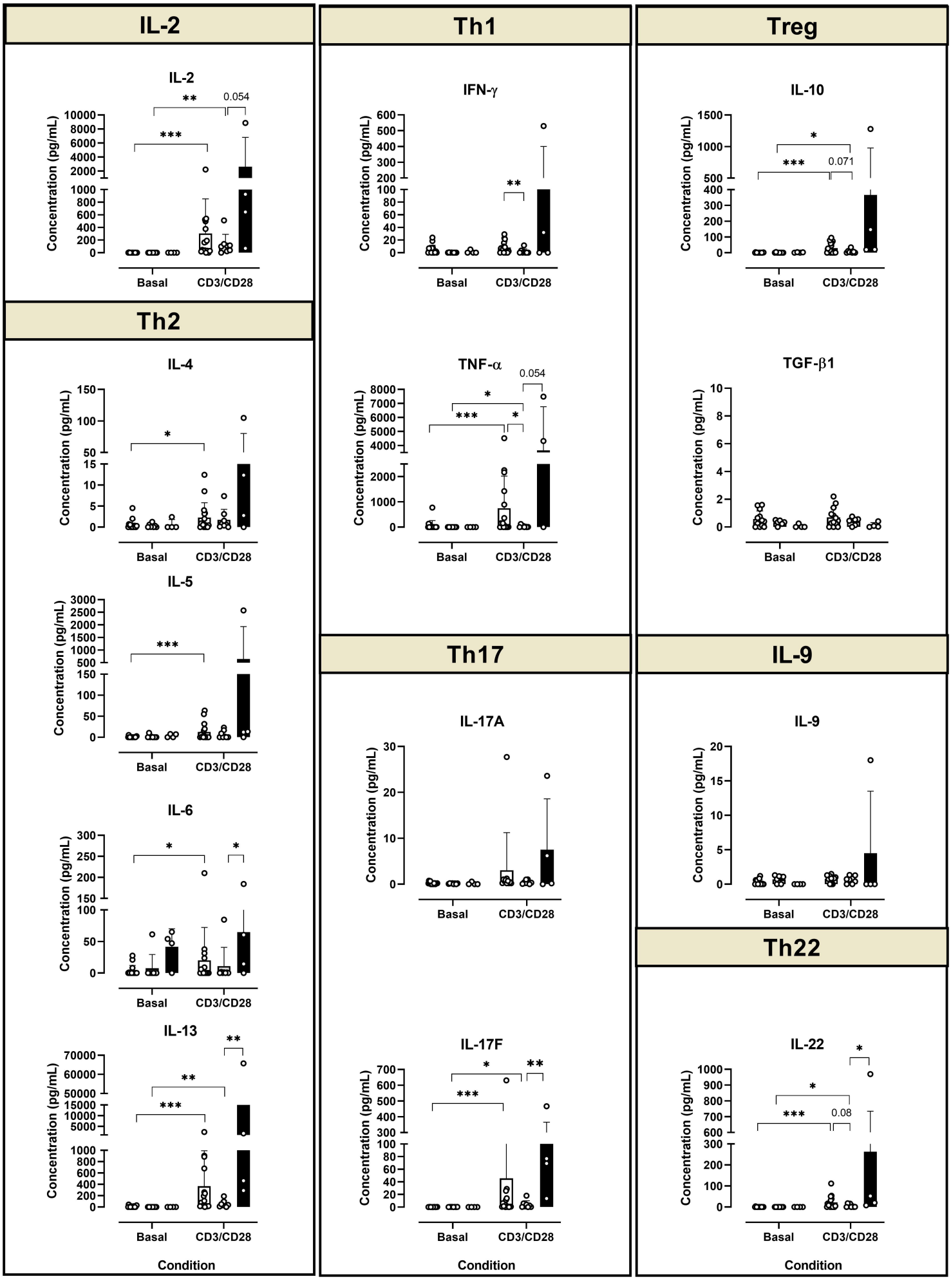
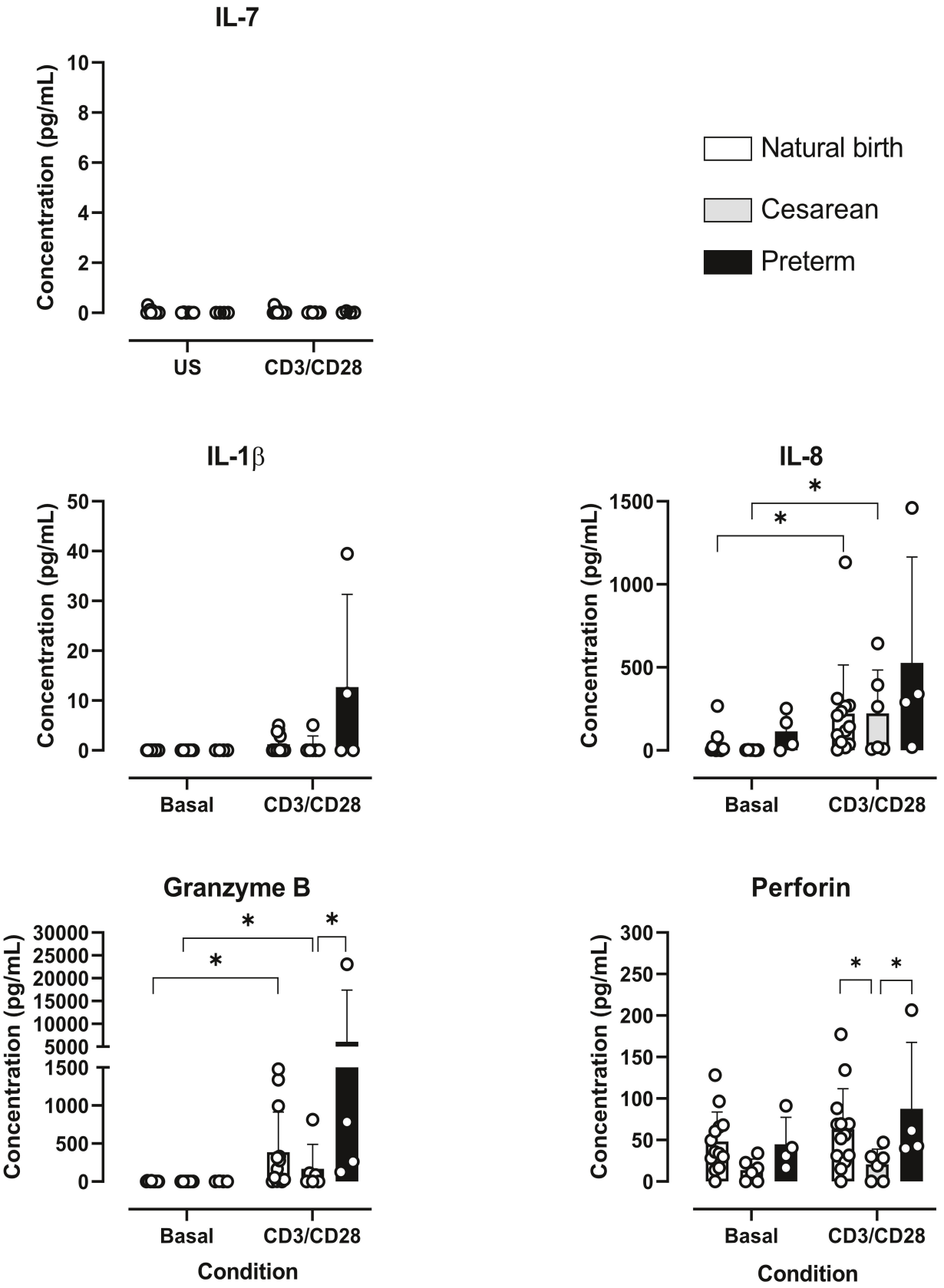
Cesarean
Preterm


A
Natural birth
Cesarean
Preterm
0
10^3
10^4
10^5
CFSE
CD3+CD28
Basal
Unstained


B
Percentage of division on CD4+ T cells
% CD4+ T cells
100
80
60
40
20
0
*
Basal
CD3/CD28
Condition
Natural birth
Cesarean
Preterm


IL-2
IL-2
Concentration (pg/mL)
Basal
CD3/CD28
Th1
IFN-γ
TNF-α
Treg
IL-10
TGF-β1
Th2
IL-4
IL-5
IL-6
IL-13
Th17
IL-17A
IL-17F
IL-9
IL-9
Th22
IL-22
Condition
Natural birth
Cesarean
Preterm

Innate cytokines and cytotoxic molecules