CD71+ Erythroid Cells from Neonates Born to Women with Preterm Labor Regulate Cytokine and Cellular Responses

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Summary sentence: Neonatal CD71+ erythroid cells regulate neonatal T-cell and myeloid responses and their direct contact with maternal mononuclear cells induces a pro-inflammatory response.

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Abbreviations

CBMC: Cord blood mononuclear cells

CCR4: C-C chemokine receptor type 4

CD71: Transferrin receptor

CSF1: Colony stimulating factor 1

CXCL5: C-X-C motif chemokine 5

EGF: Epidermal growth factor

HKLM: Heat-killed *Listeria monocytogenes*

IFNγ: Interferon gamma

IL: Interleukin

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PTL: Spontaneous preterm labor

PTNL: Preterm without labor

TGFβ: Transforming growth factor beta

TIL: Spontaneous term labor

TNFα: Tumor necrosis factor alpha

TNL: Term without labor

Abstract

Neonatal CD71+ erythroid cells are thought to have immunosuppressive functions. Recently, we demonstrated that CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm labor are reduced to levels similar to those of term neonates; yet, their functional properties are unknown. Herein, we investigated the functionality of CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm or term labor. CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm labor displayed a similar mRNA profile to that of those from term neonates. The direct contact between preterm or term neonatal CD71+ erythroid cells and maternal mononuclear immune cells, but not soluble products from these cells, induced the release of pro-inflammatory cytokines and a reduction in the release of TGFβ. Moreover, PTL-derived neonatal CD71+ erythroid cells: 1) modestly altered CD8+ T cell activation; 2) inhibited conventional CD4+ and CD8+ T-cell expansion; 3) suppressed the expansion of CD8+ regulatory T cells, as did those from neonates born to women with TIL; 4) regulated cytokine responses mounted by myeloid cells in the presence of a microbial product; and 5) indirectly modulated T-cell cytokine responses. In conclusion, neonatal CD71+ erythroid cells regulate neonatal T-cell and myeloid responses and their direct contact with maternal mononuclear cells induces a pro-inflammatory response. These findings provide insight into the biology of neonatal CD71+ erythroid cells during the physiologic and pathologic processes of labor.

Introduction

Neonates are highly susceptible to infection, which can result in long-term developmental disorders and even death [1, 2]. This susceptibility is partially due to the rapid transition from the sheltered environment *in utero* to the outside world, which results in exposure of the neonatal immune system to commensal organisms and pathogens [1]. The critical nature of this period is exacerbated by Th_2-skewed adaptive immunity [3, 4] and a reliance on transferred maternal antibodies [5], resulting in a dependence on innate immune mechanisms for protection [6, 7]. However, neonatal innate immune cells such as neutrophils [8-11], monocytes [12, 13], and dendritic cells [14] are also limited in their responses compared to adult cells. This immunosuppressed state has disadvantages such as predisposing newborns to severe infection and weakening their response to vaccination [2, 15]. Particularly, preterm neonates are at a higher risk for infection than term neonates since a severe state of immunosuppression is observed at earlier gestations [16].

Neonatal immunosuppression has been attributed to the presence of circulating nucleated erythroid cells [17-19]. Such cells undergo expansion in mid-gestation and persist throughout pregnancy in mice [18]. Nucleated erythroid cells are maintained in the circulation throughout the neonatal period and diminish as age progresses in humans [20] and in mice [19, 21]. Nucleated erythroid cells express the general erythrocyte marker glycophorin A (or CD235a) [19, 21-23] as well as the transferrin receptor CD71, an antigen that is lost upon conversion to mature erythrocytes [24]. Previous studies indicated that CD71+ erythroid cells are partially responsible for immunosuppression of the neonatal immune system [21], and that a

reduction in the number and/or functionality of these cells is observed in preterm newborns [25]. A follow-up study claimed, however, that these reticulocytes have a limited role in reducing inflammation driven by microbial colonization [26]. Recently, we demonstrated that the number and frequency of CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm labor are similar to term neonates, but lower than those born to women who delivered preterm in the absence of labor [27]. The processes of preterm and term labor are associated with inflammation in the mother and at the maternal-fetal interface [28-31]; therefore, we suggested that the reduction of neonatal CD71+ erythroid cells was associated with the physiologic (term labor) and pathologic (preterm labor) termination of pregnancy [27]. Yet, whether CD71+ erythroid cells from neonates born to mothers who underwent spontaneous preterm labor are functionally distinct from CD71+ erythroid cells from those born at term is unknown.

The aims of this study were to determine whether CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm labor display a different mRNA profile compared to those from term neonates, and whether their combination with maternal mononuclear cells can regulate the release of cytokines through soluble factors and/or direct contact. In addition, we investigated whether the depletion of CD71+ erythroid cells from neonates born to women who underwent spontaneous preterm labor can regulate neonatal innate and adaptive immune responses, and compared such responses to those from women who underwent the physiologic process of term labor.

Materials and Methods

Human subjects, clinical specimens, and definitions

Umbilical cord blood samples were obtained at the Detroit Medical Center, Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US 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 these institutions. All participating women provided written informed consent. A total of 126 umbilical cord samples were obtained from neonates born to women who delivered at term with (TIL, n=46) or without (TNL, n=20) spontaneous labor, or preterm with (PTL, n=42) or without (PTNL, n=18) spontaneous labor. Subsets of these samples were used separately in different experiments since the number of CD71+ erythroid cells is limited in human cord blood. The demographic and clinical characteristics of the four groups of women are shown in Table 1. The fetal inflammatory response was assessed by histological examination of the placenta and the umbilical cord (Table 1). Only a few cases presented mild or moderate fetal inflammatory responses in the TIL and PTL groups (Table 1). No evidence of fetal inflammatory response was observed in the control groups (TNL and PTNL) (Table 1). Preterm birth was defined as delivery before 37 weeks of gestation, and term birth was defined as delivery at or after 37 weeks of gestation. Umbilical cord blood was collected at birth in ethylene diamine tetra-acetic acid (EDTA)-containing blood collection tubes by venipuncture of the umbilical vein and

then transported to the laboratory for immediate use. Time-matched maternal blood samples were also collected for Transwell co-culture assays.

Isolation of umbilical cord CD71+ erythroid cells

Umbilical cord blood was collected from neonates born to women from each study group [PTNL (n=8), PTL (n=10), TNL (n=10), and TIL (n=10)]. Mononuclear cells were isolated from cord blood samples by density gradient using Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden), following the manufacturer's instructions. Cells were collected from the mononuclear layer of the density gradient, washed with 1X phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY) and re-suspended in MACS buffer (BSA 0.5% [Sigma-Aldrich, St. Louis, MO], EDTA 2 mmol [Life Technologies] and 1X PBS). Depletion of CD45-positive and CD15-positive cells was performed using CD45 and CD15 microbeads (Miltenyi Biotec, San Diego, CA), following the manufacturer's instructions. CD15 microbeads were used in addition to CD45 microbeads in order to ensure complete depletion of neutrophils. Briefly, mononuclear cells were labeled with CD45 and CD15 microbeads and depleted by positive selection using LS columns (Miltenyi Biotec) and a magnetic MACS separator (Miltenyi Biotec). The unbound mononuclear cells were then collected, washed in MACS buffer and centrifuged at 500 X g for 5 min, and depletion was confirmed by flow cytometry. The isolation of CD71+ erythroid cells was then performed as above using a biotin-conjugated CD71 antibody (BD Biosciences, San Jose, CA) and streptavidin-linked magnetic beads (Miltenyi Biotec) for positive selection with MACS columns. CD71+ cells were then manually counted

using a hemocytometer, followed by centrifugation at 500 X g for 5 min. Purity was assessed by flow cytometry using anti-CD3, anti-CD235a, and anti-CD71 antibodies (BD Biosciences). Cord blood CD71+ erythroid cells were identified as CD3-CD235a+CD71+ cells and their purity was greater than 90% after each isolation. Finally, CD71+ erythroid cells were re-suspended in 400µL of RLT Lysis buffer (Qiagen, Germantown, MD) and stored at -80°C until RNA isolation.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from the previously isolated CD71+ cells using Qiagen RNeasy mini kits (Qiagen), following the manufacturer's instructions. RNA concentrations and purity were assessed with the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and RNA integrity was evaluated with the Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE). Complementary (c)DNA was synthesized using the RT^2 First Strand Kit (QIAGEN). The RT^2 Profiler™ Human Cancer, Inflammation and Immunity Crosstalk PCR array (Cat#PAHS-181Z, Qiagen) included the genes listed in Supplementary Table 1. This array was used for mRNA expression profiling and performed using RT^2 SYBR Green ROX qPCR MasterMix (QIAGEN) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA).

Transwell co-culture assay

Umbilical cord blood was collected from neonates born to women from each study group [PTNL (n=10), PTL (n=11), TNL (n=10), and TIL (n=14)]. Time-matched peripheral blood samples from the mother of each neonate were also obtained. Maternal peripheral blood mononuclear cells (PBMCs) and umbilical cord CD71+ erythroid cells were isolated as described above. Co-culture experiments were performed using 0.4μm pore Transwell inserts (Corning Inc., Corning, NY) in 24-well plates (Corning Inc.). Maternal PBMCs were re-suspended in RPMI 1640 culture medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic (Life Technologies) at a concentration of 5 X 10^5 cells/600μL and placed in the bottom well of the Transwell plate. Umbilical cord CD71+ erythroid cells were re-suspended in RPMI 1640 culture medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic at a concentration of 5 X 10^5 cells/100 μL and either placed in the top well of the Transwell plate, or in the bottom well together with maternal PBMCs. Umbilical cord CD71+ erythroid cells (5 X 10^5 cells/100μL) were also placed in the top well of the Transwell plate alone. Transwell plates were incubated at 37°C with 5% CO_2 for 24 hrs. Each experiment was performed in triplicates. Following incubation, the cells and conditioned media in the upper and bottom wells were collected by gently pipetting to re-suspend the cells and transferred to sterile 1.5mL microcentrifuge tubes separately. The cell suspensions were then centrifuged at 6000 X g for 5 min. After centrifugation, supernatants were gently collected and stored at -80°C until use.

Multiplex Immunoassay

The concentrations of IFNγ, IL-10, IL-12p70, IL-17A, IL-1β, IL-2, IL-5, IL-6, and TNFα in Transwell co-culture supernatants were measured with the Human U-PLEX Multiplex Assay (CAT#K15067L-2; Meso Scale Discovery, Rockville, MD), according to the manufacturer's instructions. Plates were read using the SECTOR 2400 Imager (Meso Scale Discovery). Standard curves were generated and the assay values of the samples were interpolated from the curves. The sensitivities of the assays were: 2.9pg/mL (IFNγ), 0.2pg/mL (IL-10), 1.6pg/mL (IL-12p70), 1.2pg/mL (IL-17A), 0.3pg/mL (IL-1β), 1.8pg/mL (IL-2), 0.5pg/mL (IL-5), 0.8pg/mL (IL-6), and 1.1pg/mL (TNFα). Values for IL-6, IL-1β, TNFα, and IL-10 were reported since the remaining cytokines were below detection limits. The concentration of TGFβ in Transwell co-culture supernatants was measured using the Human TGFβ Kit (Cat#K151IUC-2; Meso Scale Discovery), according to the manufacturer's instructions. Plates were read using the SECTOR 2400 imager. A standard curve was generated and the assay values of the samples were interpolated from the curve. The TGFβ assay sensitivity was 0.1pg/mL.

***Ex vivo* activation of T cells and myeloid cells**

Umbilical cord blood mononuclear cells (CBMCs) were isolated from neonates born to women who underwent spontaneous preterm (PTL, n=14) or term (TIL, n=14) labor by density gradient, as described above. CD71+ erythroid cells were depleted or mock depleted from CBMCs using anti-CD71 microbeads (Miltenyi

Biotec) or anti-mouse IgG2a microbeads (Miltenyi Biotec), respectively. The efficiency of CD71 depletion was > 99% in all cases. These mononuclear cells are hereafter referred to as "CD71-depleted" or "mock-depleted." The CD3+ T-cell population was not affected by the depletion of CD71+ erythroid cells (Supplementary Figure 1). CD71-depleted and mock-depleted mononuclear cells were re-suspended in RPMI 1640 medium supplemented with 10% human AB serum (Cat#35-060-CI; Corning, Tewksbury, MA) and 1% penicillin/streptomycin antibiotics at a concentration of 1 X 10^7 cells/mL. To determine direct T-cell activation, CD71-depleted or mock-depleted CBMCs were placed into 96-well round bottom plates (Corning) at a concentration of 1 X 10^6 cells per well and treated with or without anti-CD3 antibody (0.125μg/mL) (eBioscience, San Diego, CA) at 37°C with 5% CO_2 for 5 hrs. To determine indirect T-cell activation and cytokine expression by myeloid cells (CD11b+ cells), CD71-depleted or mock-depleted CBMCs were incubated with or without 5 X 10^4 cells/well of heat-killed *Listeria monocytogenes* (HKLM) (InvivoGen, San Diego, CA) in the presence of 10μg/mL of Brefeldin A (BD Biosciences) at 37°C with 5% CO_2 for 5 hrs. Immediately after incubation, expression of activation markers (CD69, CD62L, CD25, CD28, CD95, and HLA-DR) by T cells and expression of cytokines (TNFα, IL-6, IFNγ, IL-10, and IL-4) by T cells and myeloid cells were assessed by flow cytometry.

Ex vivo expansion of neonatal conventional and regulatory T cells

Umbilical cord blood mononuclear cells (CBMCs) were isolated from neonates born to women who underwent spontaneous preterm (PTL (n=8) or term (TIL, n=10)

labor by density gradient, as described above. CD71-depleted and mock-depleted CBMCs were also obtained, as previously described. These cells were then re-suspended in RPMI 1640 medium supplemented with 10% human AB serum and 1% penicillin/streptomycin antibiotic at a concentration of 2 X 10^6 cells/mL. CD71-depleted and mock-depleted CBMCs were placed into 96-well round bottom plates at a concentration of 2 X 10^5 cells/well and treated with or without anti-CD3 (0.2µg/mL) (eBioscience) and anti-CD28 (1µg/mL) (eBioscience) antibodies. Next, cells were cultured for 4 days at 37°C with 5% CO_2. Immediately after incubation, cells were collected for immunophenotyping of conventional (CD4+ and CD8+ T cells) and regulatory T cells CD4+ (CD4+CD25+Foxp3+ T cells or CD8+ CD8+CD25+FoxP3+ T cells) by flow cytometry. The total numbers of conventional and regulatory T cells were determined using CountBright Absolute Counting Beads (Molecular Probes, Life Technologies, Eugene, OR, USA).

Flow cytometry

Immediately after *ex vivo* experiments, cells were retrieved by gentle resuspension in their conditioned media and transferred to FACS tubes (Corning Life Sciences, Durham, NC). Remaining adherent cells were detached from the culture wells by incubating with 100µL of 10mM of EDTA (Life Technologies) for 10 min. Cells were then washed with 1X PBS and stained with the BD Horizon Fixable Viability Stain 510 dye (BD Biosciences), prior to incubation with extracellular monoclonal antibodies. The cells were washed in 1X PBS and incubated with 20µL of human FcR blocking reagent (Miltenyi Biotec) in 80µL of BD FACS stain buffer

(Cat#554656; BD Biosciences) for 10 min at 4°C. Next, the cells were incubated with extracellular fluorochrome-conjugated anti-human monoclonal antibodies for 30 min at 4°C in the dark (Supplementary Table 2). After extracellular staining, the cells were washed with 1X PBS and immediately fixed and permeabilized using either the BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Biosciences) or the FoxP3 Transcription Factor Fixation/Permeabilization solution (Cat#00-5523-00; eBioscience). Following fixation and permeabilization, the cells were washed with 1X BD Perm/Wash Buffer (BD Biosciences) or 1X FoxP3 Permeabilization Buffer (eBioscience), resuspended in 50μL of the same buffer, and stained with intracellular or intranuclear antibodies for 30 min at 4°C in the dark (Supplementary Table 2). Stained cells were then washed with 1X permeabilization buffer, resuspended in 0.5mL of BD FACS stain buffer, and acquired using the BD LSR Fortessa Flow Cytometer (BD Bioscience) and BD FACSDiva 6.0 software (BD Bioscience). The analysis was performed and the figures were generated using FlowJo v10 software (FlowJo, Ashland, OR).

Statistical Analysis

Statistical analyses were performed using SPSS v.19.0 software (SPSS Inc., IBM Corporation, Armonk, NY) or the R package. For qRT-PCR arrays, negative ΔCt values were determined using multiple reference genes (*Actb*, *Gapdh*, and *Rplp0*) averaged within each sample to determine gene expression levels. Negative ΔCt values were modeled using linear models with coefficient significance evaluated via moderated t-tests, as implemented in the limma package of Bioconductor [32]. With

this approach, information about the gene variance is borrowed across genes leading to a more robust statistical test. Significance p-values were adjusted across the 26 genes using the False Discovery Rate method. A heat map was created by subtracting the mean gene expression of PTNL samples from the expression of each individual sample for all differentially regulated transcripts. A p-value ≤ 0.05 and a q-value (adjusted p-value) ≤ 0.1 were considered statistically significant. Normality of data was tested using the Shapiro-Wilk test. The Wilcoxon signed rank paired test was used when comparing the same sample before and after treatment, and the Mann-Whitney U-test was used for comparisons between different samples. A p-value ≤ 0.05 was considered statistically significant.

Results

PTL-derived neonatal CD71+ erythroid cells display a similar mRNA profile to that of those from term neonates

We first compared the mRNA expression of inflammation-related genes between PTL- and TIL-derived neonatal CD71+ erythroid cells (Figure 1A). Differences between gestational age-matched non-labor controls were also determined (PTNL and TNL) (Figure 1A). Our data revealed that PTL-derived neonatal CD71+ erythroid cells had a similar mRNA profile compared to those from TIL and TNL controls (Figure 1B, Supplementary Figure 2). However, the mRNA profile of PTNL-derived neonatal CD71+ erythroid cells was modestly different compared to those from both TIL and TNL (Figure 1B, Supplementary Figure 2). The transcripts differentially expressed between the PTNL and TIL/TNL groups were the following: C-C motif chemokine ligand 5 (*Ccl5*), C-X-C motif chemokine ligand 5 (*Cxcl5*), C-X-C chemokine receptor type 2 (*Cxcr2*), and transforming growth factor beta 1 (*Tgfb1*) (Figure 1B, Supplementary Figure 2). No statistical differences were observed between TIL- and TNL-derived neonatal CD71+ erythroid cells (Supplementary Figure 2). These results show that PTL-derived neonatal CD71+ erythroid cells display a similar mRNA profile to that of those from term neonates.

The direct contact between PTL- and TIL-derived neonatal CD71+ erythroid cells and maternal mononuclear immune cells alters the release of pro- and anti-inflammatory cytokines

It was previously suggested that neonatal splenocytes suppress the activation of adult cells in a co-culture system, which was likely mediated by CD71+ erythroid cells [21]. CD71+ erythroid cells are rarely present in the maternal circulation; yet, they seem to be a source for circulating cell-free fetal DNA [33, 34]. Therefore, we evaluated whether the direct contact between neonatal CD71+ erythroid cells and maternal PBMCs could regulate cytokine release (Figure 2A). Physiologically, it is also likely that umbilical cord CD71+ erythroid cells or maternal PBMCs modulate immune responses through soluble factors; therefore, these cells were co-cultured in a Transwell system (Figure 2A). There were striking differences in cytokine release between direct contact (referred to as “combined”) and Transwell co-cultures of neonatal CD71+ erythroid cells and maternal PBMCs (Figures 2B-2D and 3A&B). The combination of PTL- or TIL-derived neonatal CD71+ erythroid cells and maternal PBMCs consistently increased the release of the pro-inflammatory cytokines IL-6 (Figure 2B), IL-1β (Figure 2C), and TNFα (Figure 2D) compared to their Transwell co-cultures. The combination of PTNL-derived neonatal CD71+ erythroid cells and maternal PBMCs also increased the release of IL-6 (Figure 2B), IL-1β (Figure 2C), and TNFα (Figure 2D) compared to their Transwell co-cultures. However, the combination of TNL-derived neonatal CD71+ erythroid cells and maternal PBMCs solely increased the release of TNFα compared to their Transwell co-cultures (Figure 2D). Interestingly, the combination of PTNL-, PTL-, TNL-, and TIL-derived neonatal

CD71+ erythroid cells and maternal PBMCs reduced the release of TGFβ compared to their Transwell co-cultures (Figure 3A). The combination of PTL-derived neonatal CD71+ erythroid cells and maternal PBMCs increased the release of IL-10 compared to maternal PBMCs alone (Figure 3B), but no other differences were observed. It is worth mentioning that the concentration of cytokines released by neonatal CD71+ erythroid cells was minimal (Figures 2B-2D and 3A&B). These data indicate that the direct contact, but not soluble products, between PTL- and TIL-derived neonatal CD71+ erythroid cells and maternal mononuclear cells induces a pro-inflammatory response by increasing the release of pro-inflammatory cytokines and reducing the release of the anti-inflammatory cytokine TGFβ.

Since PTL is our study group (pathologic process of labor) and TIL is its control (physiologic process of labor), and the combination of neonatal and maternal cells from these groups display similar cytokine responses (Figures 2 and 3), we hereafter focused on investigating functional differences between the PTL- and TIL-derived neonatal CD71+ erythroid cells.

PTL-derived neonatal CD71+ erythroid cells modestly alter CD8+ T-cell activation

A previous report showed that the depletion of CD71+ erythroid cells unleashes the activation of neonatal immune cells, suggesting that these reticulocytes have immunosuppressive functions [21]. Therefore, CD71+ erythroid cells were depleted from cord blood samples collected from the placentas of women who underwent PTL or TIL. CD71+ erythroid cells were depleted or mock depleted

from CBMCs and cultured with or without anti-CD3, which causes T-cell activation [35] (Figure 4A). As expected, incubation with anti-CD3 increased the expression of CD69 while reducing the expression of CD62L by T cells (Figures 4B-E). The activation of T cells was evaluated by flow cytometry. Depletion of CD71+ erythroid cells from CBMCs reduced the frequency of stimulated TIL- and PTL-derived neonatal CD8+ T cells expressing the early activation marker CD69 [36, 37] (Figure 4B), but did not alter the frequency of CD4+CD69+ T cells (Figure 4C).

Depletion of CD71+ erythroid cells from CBMCs reduced the frequency of stimulated PTL-derived neonatal CD8+ T cells expressing CD62L, a surface marker that is downregulated upon late T-cell activation [38] (Figure 4D), but no changes were observed in the frequency of CD4+CD62L+ T cells (Figure 4E). The expression of CD25, CD28, and CD95 by neonatal T cells was not significantly altered by the depletion of CD71+ erythroid cells (data not shown). These data suggest that PTL-derived neonatal CD71+ erythroid cells modestly alter CD8+ T cell responses upon T-cell activation.

PTL-derived neonatal CD71+ erythroid cells inhibit conventional CD4+ and CD8+ T-cell expansion

We next assessed the effect of neonatal CD71+ erythroid cells on neonatal T-cell responses. CBMCs were depleted or mock depleted of CD71+ erythroid cells and the expansion of conventional CD4+ and CD8+ T cells was determined (Figure 5A). Depletion of CD71+ erythroid cells from PTL-derived cord blood samples resulted in an expansion of both neonatal CD4+ (Figure 5B) and CD8+ (Figure 5C) T

cells regardless of T-cell activation. These results demonstrate that PTL-derived neonatal CD71+ erythroid cells can inhibit the expansion of conventional CD4+ and CD8+ T cells.

PTL- and TIL-derived neonatal CD71+ erythroid cells suppress the expansion of CD8+ regulatory T cells

A previous report suggested that CD71+ erythroid cells have immunosuppressive properties [21]. Since immunosuppression during pregnancy is largely mediated by regulatory T cells [39-44], we determined whether the depletion of CD71+ erythroid cells had an effect on the *ex vivo* expansion of regulatory T cells (Figure 5A). Depletion of CD71+ erythroid cells did not significantly alter the number of PTL-derived CD4+ regulatory T cells; yet, an expansion of such cells was observed upon T-cell activation via CD3/CD28 stimulation (Figure 5D). In contrast, depletion of CD71+ erythroid cells induced the expansion of CD8+ regulatory T cells, even in the absence of T-cell activation via CD3/CD28 stimulation (Figure 5E). Nevertheless, such an expansion was greater upon CD3/CD28 stimulation (Figure 5E). These results demonstrate that PTL- and TIL-derived neonatal CD71+ erythroid cells suppress the expansion of CD8+ regulatory T cells, but do not have an effect on CD4+ regulatory T cells.

PTL-derived neonatal CD71+ erythroid cells regulate cytokine responses mounted by myeloid cells in the presence of microbial products

Neonatal CD71+ erythroid cells have also been implicated in the activation of myeloid cells [21]. Therefore, following the same strategy as before, CBMCs were depleted or mock depleted of CD71+ erythroid cells, stimulated with HKLM, and cytokine responses by myeloid cells were evaluated (Figure 6A). Depletion of CD71+ erythroid cells from CBMCs reduced the frequencies of PTL-derived CD11b+ myeloid cells expressing IL-6 (Figure 6C), IFNγ (Figure 6D), and IL-4 (Figure 6E) upon HKLM-stimulation. In addition, depletion of CD71+ erythroid cells reduced the frequencies of unstimulated PTL-derived CD11b+ myeloid cells expressing IFNγ (Figure 6D) and IL-4 (Figure 6E). Depletion of CD71+ erythroid cells reduced the frequency of CD11b+ myeloid cells expressing IL-6 upon HKLM stimulation (Figure 6C) as well as the expression of IL-6 (Figure 6C), IL-4 (Figure 6E), and IL-10 (Figure 6F) by unstimulated CD11b+ myeloid cells in the TIL group. These data imply that PTL-derived neonatal CD71+ erythroid cells regulate cytokine responses mounted by myeloid cells in the presence of microbial products. CD71+ erythroid cells from term neonates, however, can participate in cytokine responses in the absence of infection.

PTL-derived neonatal CD71+ erythroid cells indirectly modulate T-cell cytokine responses

In vitro stimulation with HKLM results in indirect T-cell cytokine expression by initiating innate immune responses [45]. We therefore evaluated whether CD71+ erythroid cells could regulate the expression of pro- and anti-inflammatory cytokines by PTL-derived neonatal T cells (Figure 7A). Depletion of CD71+ erythroid cells from

CBMCs consistently reduced the frequencies of HKLM-stimulated PTL-derived neonatal T cells expressing TNFα (Figure 7B), IL-6 (Figure 7C), IFNγ (Figure 7D), and IL-4 (Figure 7E). Depletion of CD71+ erythroid cells from CBMCs did not change the expression of IL-10 on HKLM-stimulated PTL-derived neonatal T cells (Figure 7F). In addition, depletion of CD71+ erythroid cells reduced the expression of IL-6 (Figure 7C), IFNγ (Figure 7D), and IL-4 (Figure 7E) by unstimulated PTL-derived neonatal T cells. Depletion of CD71+ erythroid cells also reduced the expression of TNFα (Figure 7B), IL-4 (Figure 7E), and IL-10 (Figure 7F) by unstimulated TIL-derived neonatal T cells. Together, these data suggest that PTL-derived neonatal CD71+ erythroid cells indirectly regulate T-cell cytokine responses.

Further immunophenotyping revealed that depletion of CD71+ erythroid cells from CBMCs consistently reduced the frequency of HKLM-stimulated PTL-derived CD8+ T cells expressing TNFα, IL-6, IFNγ, and IL-4 (Supplementary Figure 3A-D). However, only the frequency of HKLM-stimulated PTL-derived CD4+ T cells expressing IL-6 and IL-4 was reduced upon CD71 depletion (Supplementary Figure 4B&D). Therefore, PTL-derived neonatal CD71+ erythroid cells indirectly regulate T-cell cytokines mainly by modulating CD8+ T-cell responses.

Discussion

Principal findings of the study: 1) PTL-derived neonatal CD71+ erythroid cells displayed a similar mRNA profile to that of those from term neonates and 2) the direct contact, but not soluble products, between preterm or term neonatal CD71+ erythroid cells and maternal mononuclear immune cells induced the release of pro-inflammatory cytokines and a reduction in the release of the anti-inflammatory cytokine TGFβ. Moreover, PTL-derived neonatal CD71+ erythroid cells: 3) modestly altered CD8+ T cell activation; 4) inhibited conventional CD4+ and CD8+ T-cell expansion; 5) suppressed the expansion of CD8+ regulatory T cells, as did those from neonates born to women with TIL; 6) regulated cytokine responses mounted by myeloid cells in the presence of a microbial product; and 7) indirectly modulated T-cell cytokine responses. Collectively, these findings show that CD71+ erythroid cells from neonates born to women with preterm labor regulate neonatal T-cell and myeloid responses and their direct contact with maternal mononuclear cells induces a pro-inflammatory response.

PTL- and TIL-derived CD71+ erythroid cells display similar mRNA profiles

The mRNA expression of *Ccl5*, *Cxcl5*, *Cxcr2*, and *Tgfb1* was upregulated in PTL-derived neonatal CD71+ erythroid cells and those from women with TIL or TNL when compared to neonatal CD71+ erythroid cells from women with PTNL. The concentrations of the chemokines CCL5 [46, 47] and CXCL5 [48-50], as well as the cytokine TGFβ [51, 52], are increased in the amniotic fluid of women undergoing the physiologic and pathologic processes of labor. In addition, the fetal membranes

surrounding the amniotic cavity express the chemokine receptor CXCR2 during the physiologic process of labor [53, 54]..Together, these data suggest that the expression of these mediators by PTL-derived neonatal CD71+ erythroid cells may participate in the inflammatory milieu that accompanies the premature process of labor. In addition, these findings support the concept that the fetal immune system undergoes a premature activation during preterm labor [55].

The direct contact between PTL- and TIL-derived neonatal CD71+ erythroid cells and maternal mononuclear immune cells induces a pro-inflammatory response

The direct contact between PTL- and TIL-derived neonatal CD71+ erythroid cells and maternal mononuclear immune cells drives a pro-inflammatory response by increasing the release of IL-6, IL-1β, and TNFα, and reducing the release of the anti-inflammatory cytokine TGFβ. These findings contrast with a previous report demonstrating that the direct contact between neonatal and adult immune cells attenuates pro-inflammatory cytokine production [21]. A possible explanation for this discrepancy is that the previous study was performed using 6-day old mice [21] and the current study included human cord blood samples from term and preterm neonates; therefore, it is likely that the function of CD71+ erythroid cells differs between species and age. The findings reported herein indicate that in the scenario where fetal CD71+ erythroid cells migrate into the maternal circulation [33, 34], their direct interaction can result in an inflammatory response, which may contribute to the inflammatory process present in women who undergo spontaneous preterm labor

[56]. The biological relevance of these findings will be clarified upon addressing how and when CD71+ erythroid cells reach the maternal circulation as well as whether such cells can localize in a specific microenvironment throughout pregnancy, and more importantly, during the process of labor. In this context, fetal CD71+ erythroid cells are a source of cell-free fetal DNA [33, 34], which concentration increases prior to the process of labor [57, 58].

PTL-derived neonatal CD71+ erythroid cells modulate direct and indirect T-cell responses

CD69 is an early marker of T-cell activation [36, 37], and our findings indicate that PTL- and TIL-derived CD71+ erythroid cells enhance early CD8+ T-cell activation through increased frequency of cells expressing this marker. A previous study demonstrated that the depletion of CD71+ erythroid cells in cord blood samples from term neonates unleashes the activation of T cells [21]. A possible explanation for this discrepancy is that the majority of our samples were obtained from African-American women, which may be different from the study population included in previous studies.

CD62L is also known as L-selectin and its enhanced expression is associated with naïve- and memory-like T-cell phenotypes, whereas a reduction in the expression of this cell adhesion molecule occurs after T-cell activation [38]. Therefore, the downregulation of CD62L is considered a marker of late T-cell activation [59]. The fact that the depletion of CD71+ erythroid cells reduced the frequency of PTL-derived CD8+ T cells expressing CD62L indicates that such

neonatal cells can also inhibit the process of late CD8+ T-cell activation. Consistently, we found that PTL-derived neonatal CD71+ erythroid cells inhibit CD4+ and CD8+ T-cell proliferation. Together, these data suggest that PTL-derived neonatal CD71+ erythroid cells can inhibit late T-cell activation and proliferation. This is relevant since neonatal T-cell activation has been closely associated with spontaneous preterm labor in cases with clinical chorioamnionitis [60]. In addition, maternal T-cell activation has been causally linked to spontaneous preterm labor and fetal death [61]. Yet, whether maternal T-cell activation indirectly causes fetal T-cell activation requires further investigation.

Our data also show that CD71+ erythroid cells from preterm neonates modulate the frequencies of cytokine-expressing neonatal T cells upon indirect T-cell stimulation. A previous report indicated that CD71+ erythroid cells from healthy term neonates suppress the expression of pro-inflammatory cytokines by activated T cells upon incubation with staphylococcal enterotoxin B [62]. A possible explanation for this discrepancy is that the previous study used cord blood samples from term neonates with unknown labor status and we used those from preterm or term neonates whose mothers underwent spontaneous preterm or term labor, respectively. Regardless, our T-cell cytokine data are consistent with the previous research indicating that preterm labor is characterized by the activation of the fetal immune system [55], which can be caused by microorganisms and/or microbial products [63-65].

PTL- and TIL-derived neonatal CD71+ erythroid cells suppress the expansion of CD8+ regulatory T cells

CD71+ erythroid cells suppress CD8+ regulatory T cells in neonates born to women who underwent spontaneous preterm or term labor. CD8+ regulatory T cells express Foxp3 and therefore seem to share phenotypical and functional characteristics with classical CD4+ regulatory T cells [66]. These regulatory cells inhibit T-cell responses (e.g. Th17 cells) *in vivo* [67] and seem to participate in the mechanisms of maternal-fetal tolerance during normal pregnancy [68]. In late pregnancy, however, maternal CD8+ regulatory T cells in the decidua regulate the timing of term parturition by IL6-mediated mechanisms [69]. In addition, maternal CD8+ regulatory T cells have been implicated in the systemic and local (i.e. the maternal-fetal interface) immune mechanisms that lead to spontaneous preterm labor [70, 71]. CD8+ regulatory T cells are induced extrathymically through antigen presentation in the neonatal period in mice [72]; however, their role in humans is unknown. Our findings provide insight into the cellular mechanisms (e.g. CD71+ erythroid cells) regulating the expansion of such immunosuppressive cells. Whether CD8+ regulatory T cells suppress effector T-cell functions in term and preterm neonates requires further investigation.

It is worth mentioning that while the depletion of neonatal CD71+ erythroid cells tended to decrease the number of TIL-derived neonatal CD4+ regulatory T cells, the depletion of such cells tended to increase the number of PTL-derived neonatal CD4+ regulatory T cells. Further research is required to demonstrate whether in the

physiologic or pathologic process of labor neonatal CD71+ erythroid cells exhibit CD4+ Treg-mediated immunosuppression.

PTL-derived neonatal CD71+ erythroid cells regulate cytokine responses by myeloid cells in the presence of a microbial product

Our findings demonstrate that PTL-derived neonatal CD71+ erythroid cells suppress the frequency of myeloid cells expressing pro-inflammatory cytokines (TNFα, IL-6, and IFNγ) and the anti-inflammatory cytokine IL-4 upon HKLM stimulation. A previous report showed that the depletion of CD71+ erythroid cells in term-derived cord blood mononuclear cells unleashes the expression of inflammatory cytokines by myeloid cells [21, 73]. Yet, our data are consistent with previous observations demonstrating that fetal monocyte activation, as assessed by the expression of IL-6, is associated with the process of labor [74]. In addition, a more recent study has demonstrated that monocytes from preterm neonates display a high phagocytic activity but an impaired activation of ERK1/2 and NF-κB pathways upon TLR stimulation [75]. Collectively, these findings suggest that PTL-derived neonatal CD71+ erythroid cells participate in the regulation of cytokine production by neonatal myeloid cells in the presence of a microbial product.

In summary, CD71+ erythroid cells from neonates born to women with preterm labor display an mRNA profile which is similar to that of those from term neonates. In addition, neonatal CD71+ erythroid cells regulate neonatal T-cell and myeloid responses and their direct contact with maternal mononuclear cells induces a pro-inflammatory response. These findings provide insight into the biology of

neonatal CD71+ erythroid cells during the physiologic and pathologic processes of labor.

Authorship

DM - contributed to the execution of the experiments, data analyses, drafting of the manuscript, and critical discussion of the findings.

RR - contributed to the conception, data analyses, and critical discussion of the findings, and contributed to the drafting of the manuscript.

RU - contributed to the execution of the experiments, critical discussion of the findings, and participated in the drafting of the manuscript.

YX – contributed to the design of the experiments, critical discussion of the findings, and participated in the drafting of the manuscript.

FVO - contributed to the execution of the experiments, critical discussion of the findings, and participated in the drafting of the manuscript.

SSH - contributed to the sample collection, critical discussion of the findings and data analyses.

NGL - conceived and designed the study, provided critical discussion of the findings, contributed to the drafting of the manuscript, and revised the final version of the manuscript.

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Disclosures

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Figure Legends

Figure 1: PTL-derived neonatal CD71+ erythroid cells display a similar mRNA profile to that of those from term neonates. **(A)** Experimental design for mRNA profiling in isolated CD71+ erythroid cells. **(B)** Heat map of differentially expressed mRNA transcripts in CD71+ erythroid cells. Red squares indicate upregulation and green squares indicate downregulation. PTNL = preterm without labor, PTL = spontaneous preterm labor, TNL = term without labor, TIL = spontaneous term labor. n=8-10 each.

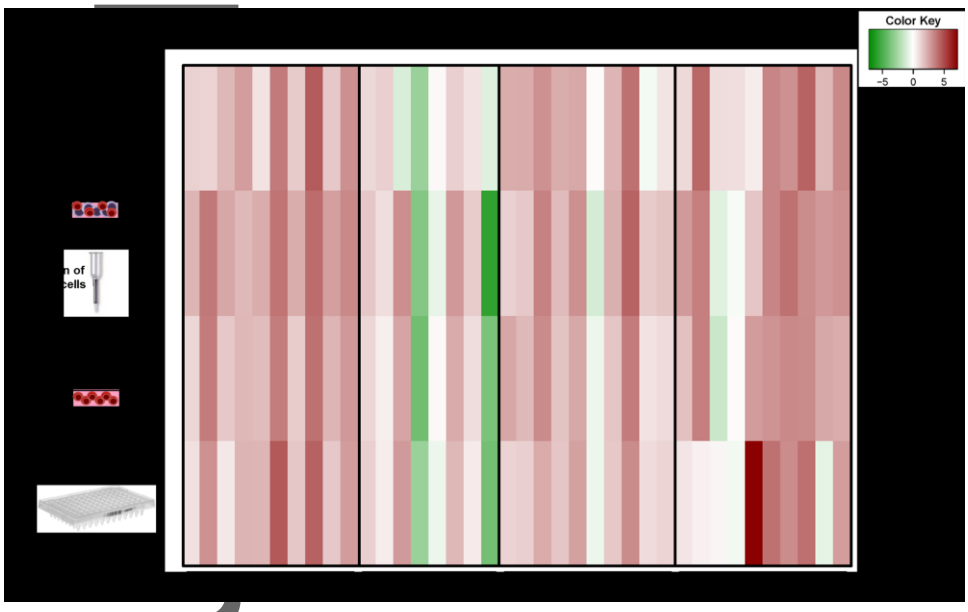

Figure 2: Direct contact between neonatal CD71+ erythroid cells and maternal PBMCs induces the release of pro-inflammatory cytokines. **(A)** Experimental design

of combined and Transwell co-cultures between neonatal CD71+ erythroid cells and maternal PBMCs. Neonatal CD71+ erythroid cells and maternal PBMCs were also cultured alone (controls). The release of IL-6 **(B)**, IL-1β **(C)**, and TNFα **(D)** was determined by ELISA. PTNL = preterm without labor, PTL = spontaneous preterm labor, TNL = term without labor, TIL = spontaneous term labor. n = 10-14 each.

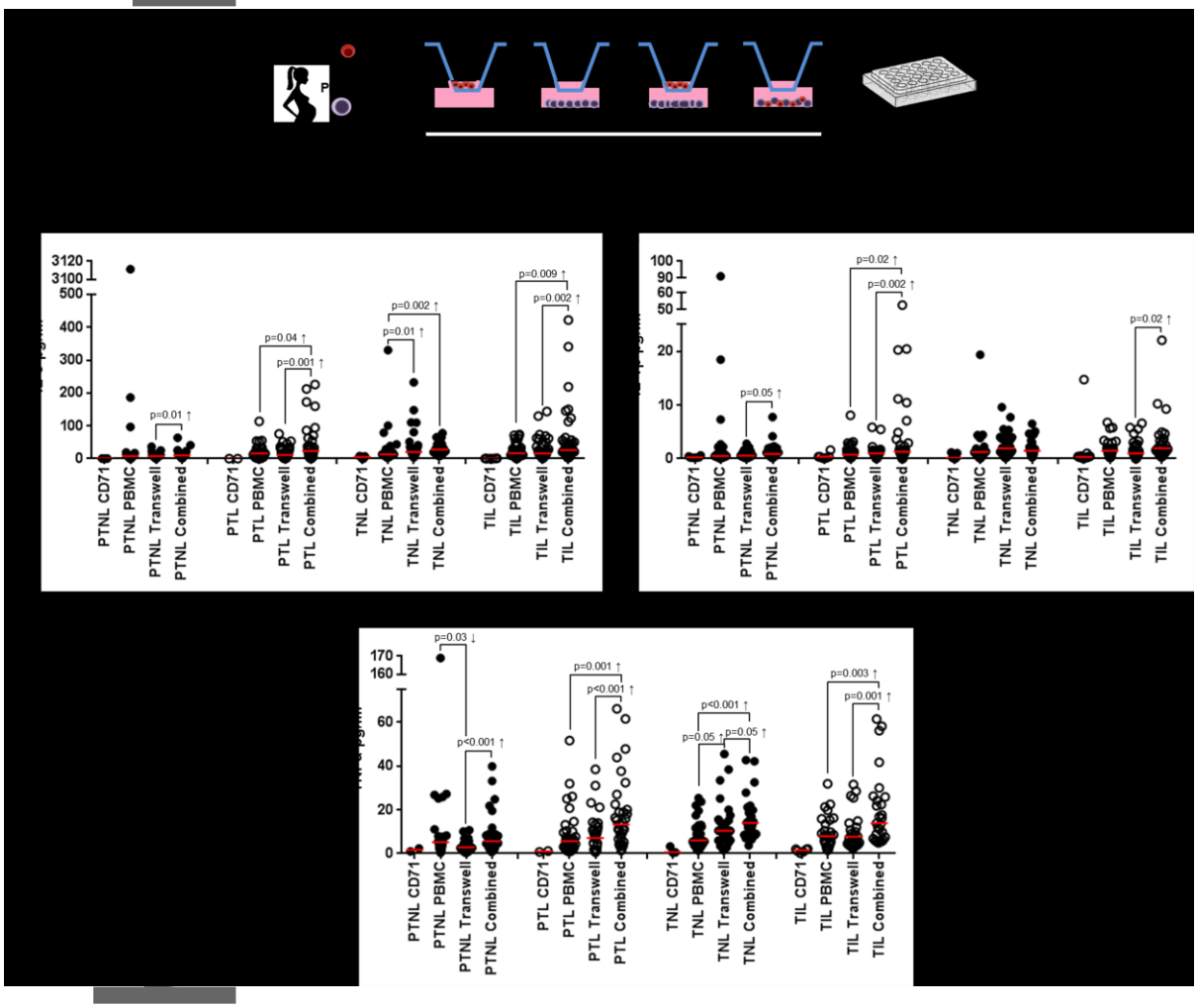

Figure 3: Direct contact between neonatal CD71+ erythroid cells and maternal PBMCs reduces the release of TGFβ but not IL-10. The release of TGFβ **(A)** and IL-10 **(B)** was determined by ELISA. PTNL = preterm without labor, PTL = spontaneous

preterm labor, TNL = term without labor, TIL = spontaneous term labor. n = 10-14 each.

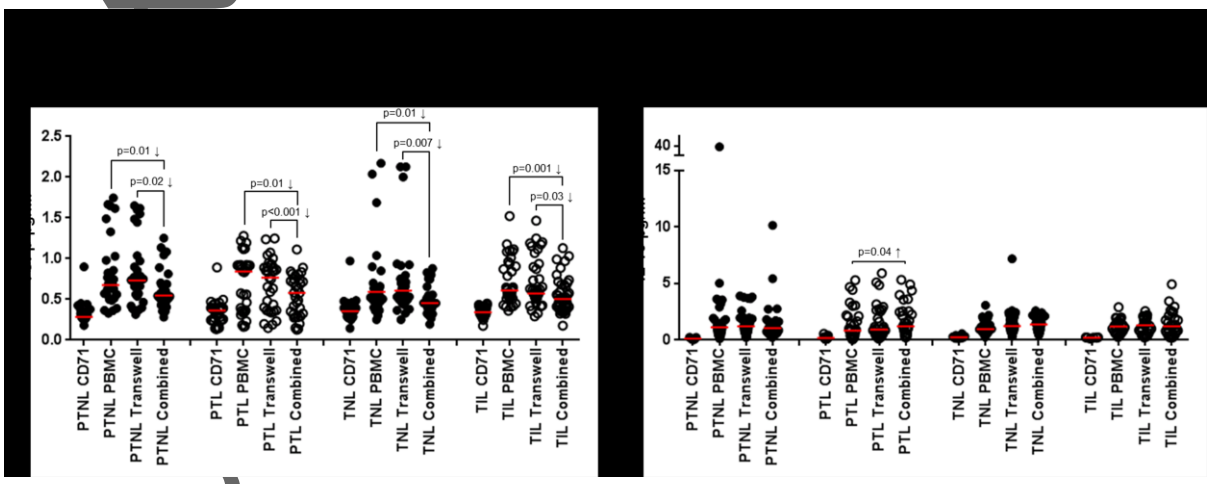

Figure 4: PTL-derived neonatal CD71+ erythroid cells modestly alter CD8+ T-cell activation. **(A)** Cord blood mononuclear cells were CD71-depleted or mock-depleted. Experimental design and flow cytometry gating strategy for studying neonatal T-cell activation by CD71+ erythroid cells. Representative flow cytometry histograms show the expression of CD28, CD62L, CD25, CD69, and CD95 by T-cells from CD71-depleted (blue) or mock depleted (green or pink) cord blood samples incubated with anti-CD3. Autofluorescence controls are shown in black outline. Frequencies of CD69-expressing CD8+ **(B)** and CD4+ **(C)** T cells. Frequencies of CD62L-expressing CD8+ **(D)** and CD4+ **(E)** T cells. Orange dots represent neonatal T cells from preterm labor-derived cord blood samples and black dots represent neonatal T cells from term labor-derived cord blood samples. n = 8-10 each.

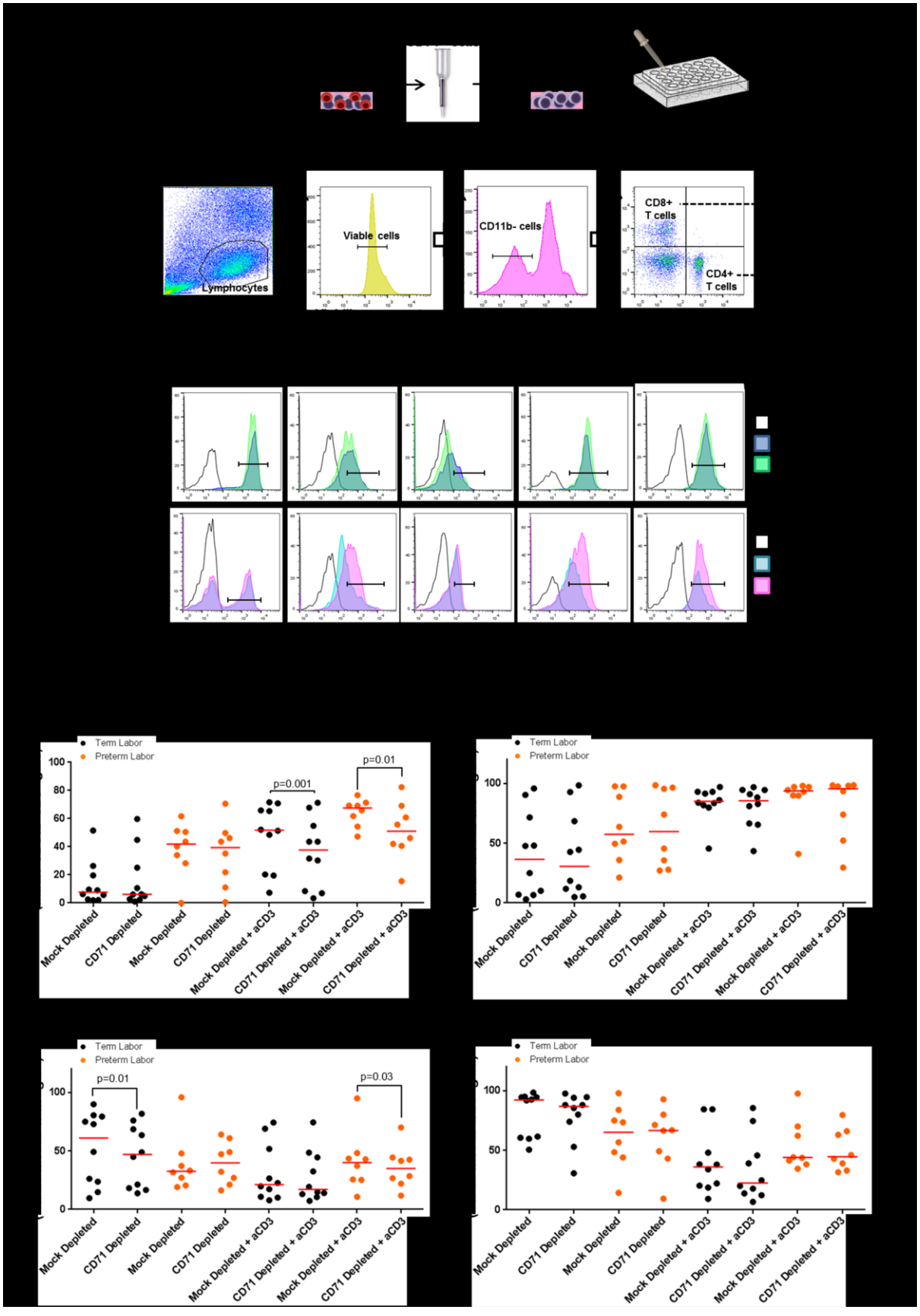

Figure 5: Neonatal CD71+ erythroid cells inhibit the expansion of conventional CD4+ and CD8+ T cells as well as CD8+ regulatory T cells**. (A)** Cord blood mononuclear cells were CD71-depleted or mock-depleted and incubated with or without anti-CD3/CD28. Experimental design and representative flow cytometry gating strategy for studying the effect of neonatal CD71+ erythroid cells on the *ex vivo* expansion of T cells. CD4+ and CD8+ regulatory T cells were identified by flow cytometry within the CD3+CD4+ and CD3+CD8+ gates, respectively. **(B)** Number of total CD4+ T cells. **(C)** Number of total CD8+ T cells. **(D)** Number of CD4+ regulatory T cells (CD4+CD25+FoxP3+ cells). **(E)** Number of CD8+ regulatory T cells (CD8+CD25+FoxP3+ cells). Orange dots represent neonatal regulatory T cells from preterm labor-derived cord blood samples and black dots represent neonatal regulatory T cells from term labor-derived cord blood samples. n = 8-10 each.

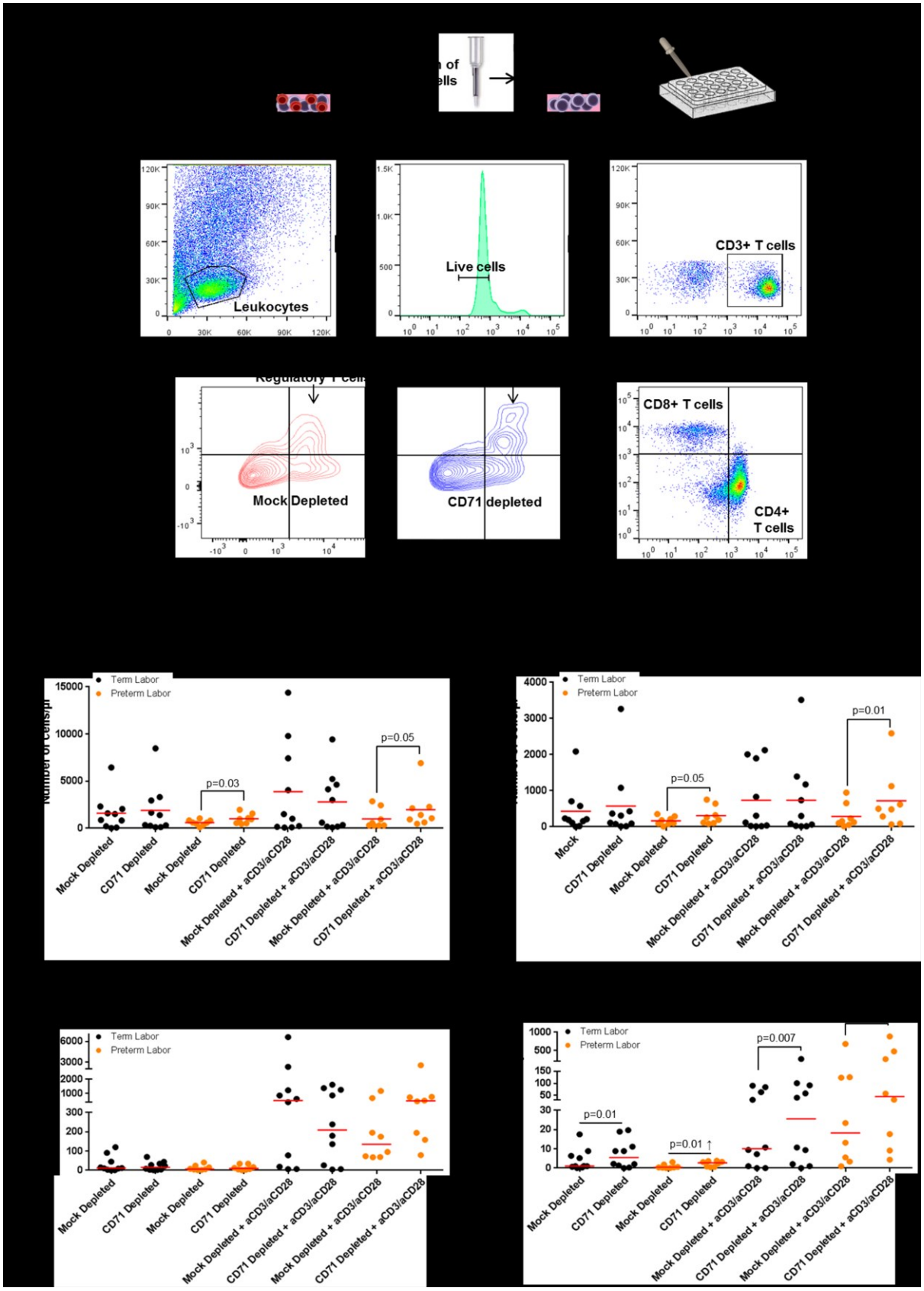

Figure 6: PTL-derived neonatal CD71+ erythroid cells regulate cytokine responses by myeloid cells in the presence of a microbial product. **(A)** Cord blood mononuclear cells were CD71-depleted or mock-depleted. Representative flow cytometry histograms show the expression of TNFα, IL-6, IFNγ, IL-4 and IL-10 by myeloid cells (CD11b+ cells) from CD71-depleted (blue) or mock depleted (purple) cord blood samples incubated with HKLM. Isotype controls are shown in black outline. Frequencies of CD11b+ myeloid cells expressing TNFα **(B)**, IL6 **(C)**, IFNγ **(D)**, IL4 **(E)**, and IL-10 **(F)**. Orange dots represent neonatal myeloid cells from preterm labor-derived cord blood samples and black dots represent neonatal myeloid cells from term labor-derived cord blood samples. n = 8-10 each.

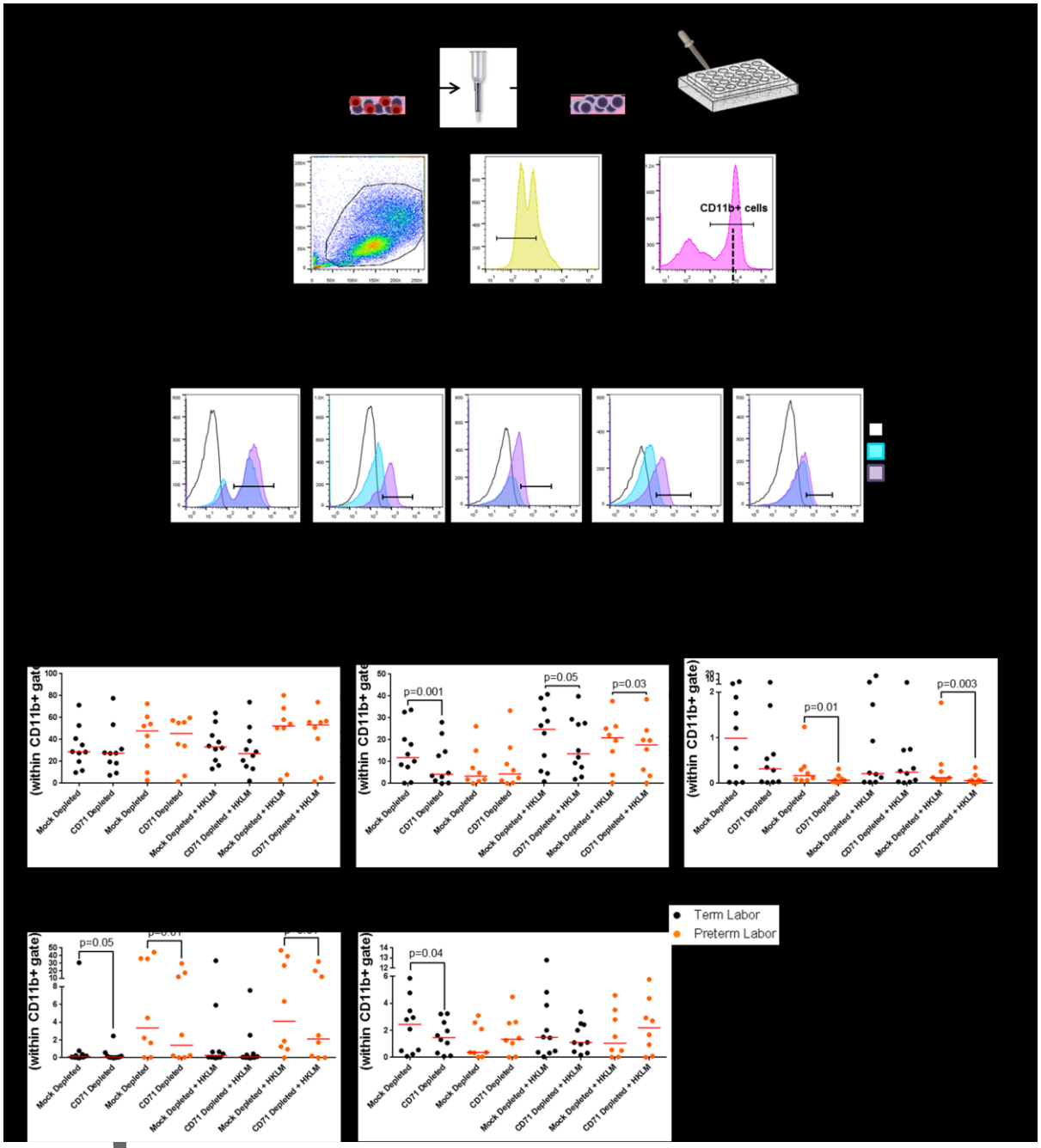

Figure 7: PTL-derived neonatal CD71+ erythroid cells indirectly modulate T-cell cytokine responses. **(A)** Cord blood mononuclear cells were CD71-depleted or mock-depleted. Experimental design and flow cytometry gating strategy for studying neonatal T-cell cytokine responses by CD71+ erythroid cells. Representative flow

cytometry histograms show the expression of TNFα, IL-6, IFNγ, IL-4 and IL-10 by T cells (CD3+ cells) from CD71-depleted (blue) or mock depleted (pink) cord blood samples incubated with HKLM. Isotype controls are shown in black outline. Frequencies of CD3+ T cells expressing TNFα **(B)**, IL6 **(C)**, IFNγ **(D)**, IL4 **(E)**, and IL-10 **(F)**. Orange dots represent neonatal T cells from preterm labor-derived cord blood samples and black dots represent neonatal T cells from term labor-derived cord blood samples. n = 8-10 each.

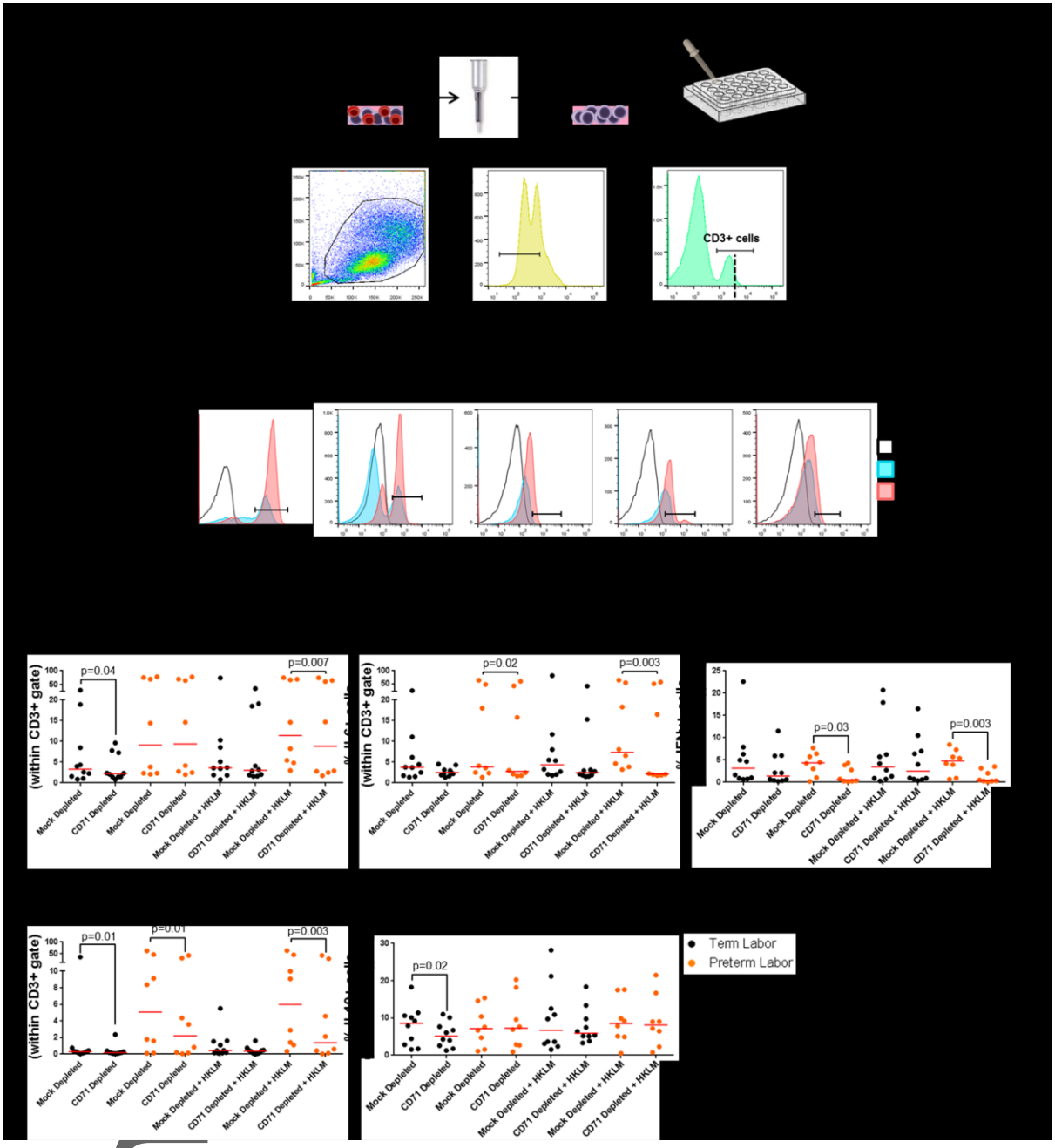

Table 1. Demographic and clinical characteristics of the samples used for study

	PTNL (n=18)	PTL (n=42)	TNL (n=20)	TIL (n=46)	p value
Age (y; median [IQR])[a]	31 (28.3-34)	23.5 (22-27.8)	25 (21-28.5)	25 (21-28)	p=0.007
Body mass index (kg/m^2; median [IQR])[a]	36.3 (25.8-39.4)	27.4 (20.5-31.1)	28.5 (22.3-33.8)	27.5 (22.5-32.6)	p=0.037
Gestational age at delivery (wk; median [IQR])[a]	36.1 (34.7-36.7)	34.8 (34-35.9)	39 (38.9-39.2)	38.9 (38.1-39.9)	p<0.001
Birth weight (g; median [IQR])[a]	2665 (2243.8-3077.5)	2255 (2040-2587.5)	3215 (2906.3-3671.3)	3080 (2852.5-3447.5)	p<0.001
Race (n[%])[b] **African-American** **Caucasian** **Other**	 13 (72.2%) 4 (22.2%) 1 (5.6%)	 37 (88.1%) 3 (7.1%) 2 (4.8%)	 19 (95%) 1 (5%) 0 (0%)	 40 (87.0%) 1 (2.2%) 5 (10.8%)	p=0.006
Primiparity (n[%])[b]	1 (5.6%)	8 (19.1%)	0 (0%)	13 (28.3%)	p=0.004
Cesarean section (n[%])[b]	18 (100%)	8 (19.1%)	20 (100%)	6 (13%)	p<0.001
Umbilical cord pathology (n[%])[b]					
Umbilical phlebitis	0 (0%)	6 (14.3%)	0 (0%)	10 (21.7%)	p=0.002
Umbilical arteritis	0 (0%)	3 (7.1%)	0 (0%)	3 (6.5%)	NS
Necrotizing funisitis	0 (0%)	0 (0%)	0 (0%)	0 (%)	NS

[a]Kruskal-Wallis test, [b]X^2 test, IQR = interquartile range